CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY
AND PHARMACEUTICAL ANALYSIS

Amidoximes as intermediates for the synthesis of potential drugs

Diploma Thesis

Hradec Králové 2015 Anastasia Katirtzi

I declare that this thesis is my original copyrighted work. All literature and other resources I used while processing are listed in the bibliography and properly cited.

In Hradec Králové 15. 5. 2015 Anastasia Katirtzi

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ABSTRACT

Charles University in Prague, Faculty of Pharmacy in Hradec Králové
Department of Pharmaceutical Chemistry and Pharmaceutical Analysis
Student: Anastasia Katirtzi
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Title of Diploma Thesis: Amidoximes as intermediates for the synthesis of potential drugs

The current thesis is focused on the *O*-acylation of pyrazine amidoximes and their cyclization to the corresponding 3,5-disubstituted- 1,2,4-oxadiazoles. 5-unsubstituted and 5-butylpyrazine amidoximes were acylated by acetic anhydride, trimethylacetic anhydride and 2,3-pyrazinecarboxylic anhydride as acylating agents in toluene. Formation of oxadiazoles was achieved in xylene, using acetic anhydride for acylation. Identification of the products was done by melting points, NMR and IR spectra, and their purity was proved by elemental analysis Furthermore, amidoximes and their derivatives were subjected to biological assay in order to evaluate their *in vitro* antifungal and antibacterial properties. Unfortunatelly, amidoximes and 1,2,4-oxadiazoles were inactive. For the esters, biological results will be available later.

ABSTRAKT

Univerzita Karlova v Praze, Farmaceutická fakulta v Hradci Králové
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Student: Anastasia Katirtzi
Školitel: doc. RNDr. Veronika Opletalová, Ph.D.
Název diplomové práce: Amidoximy jako meziprodukty pro syntézu potenciálních léčiv

Tato práce je zaměřena na *O*-acylaci amidoximů odvozených od pyrazinu a jejich cyklizaci na odpovídající 3,5-disubstituované-1,2,4-oxadiazoly. 5-nesubstituovaný a 5-butylpyrazinamidoxim byly acylovány anhydridem kyseliny octové, resp. anhydridem kyseliny trimethyloctové, v toluenu. Oxadiazoly byly připraveny zahříváním amidoximů s anhydridem kyseliny octové v xylenu. Identificace produktů byla provedena na základě teploty tání, NMR a IČ spekter a čistota ověřena elementární analýzou. U amidoximů a jejich derivátů byly dále testovány *in vitro* jejich antifungální a antibakteriální vlastnosti. Amidoximy a oxadiazoly byly bohužel neúčinné, pro ester prozatím nejsou výsledky k dispozici.

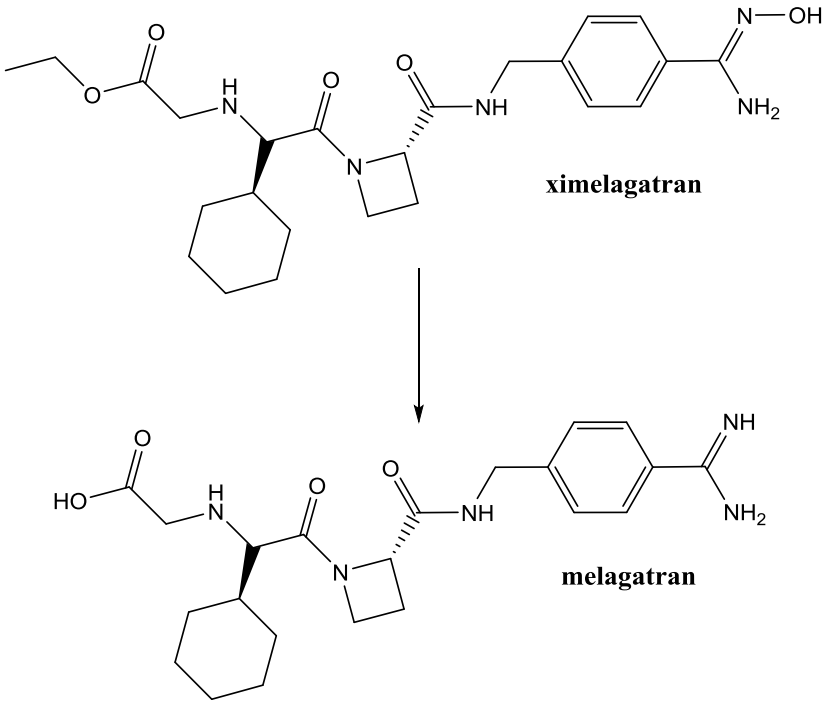
CONTENTS

Abbreviations

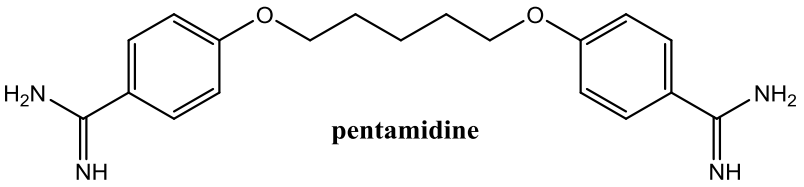
CDI	1,1′-carbonyldiimidazole
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DMF	dimethylformamide
EDC	1-ethyl-3-[(*N*,*N*-dimethylamino)propyl]carbodiimide
GP	glycogen phosphorylase
H3-antagonists	histamine H3 receptors antagonists
HBTU	*O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium
HOBt	1-hydroxy-1*H*-1,2,3-benzotriazole
HSP	heat shock proteins
IR	infra red
MAOI	monoamine oxidase inhibitors
mGlu5	metabotropic glutamate subtype 5 receptor
MW	microwave irradiation
NMR	nuclear magnetic resonance
NO	nitric oxide
NOS	nitric oxide synthase
PARP	poly(ADP-ribose)polymerase
TBAF	tetra-*N*-butylammonium fluoride
TBTU	2-(1*H*-benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium
TFAA	trifluoroacetic anhydride
THF	tetrahydrofuran

1. Introduction

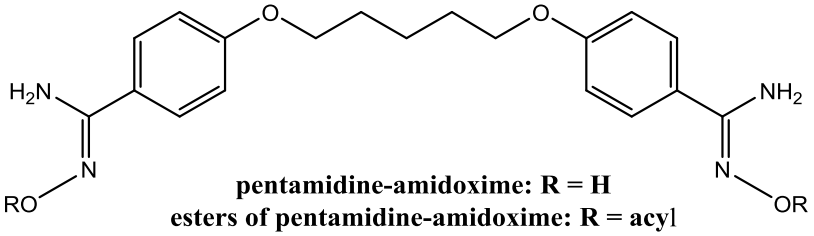
Amidoximes are very versatile compounds, and their chemical properties and biological effects have previously been reviewed [1–12]. In drug discovery and design, they have been studied as prodrugs of amidines. **Ximelagatran** is a prodrug which is converted to direct thrombin inhibitor melagatran by hydrolysis of the ester group and reduction of the amidoxime moiety. It seemed to be very promising drug since the introduction of warfarin, there have been no new anticoagulants for about 50 years Ximelagatran was used for a limited time as the preparation *EXANTA*. In 2006, it was withdrawn because of serious hepatotoxicity. Nonetheless, it is still used as an important pharmacological tool to study pathogenesis of idiosyncratic drug-induced liver injury (DILI) [13–17].



Many studies have been devoted to amidoxime-prodrugs of antiprotozoal drug **pentamidine** and its analogues [18–26]. Esters of pentamidine-amidoximes with dicarboxylic acids have been patented as potential therapeutic or prophylactic agents against cancer, leishmaniosis, trypanosomiasis, *Pneumocystis carinii* pneumonia, and malaria [27, 28].

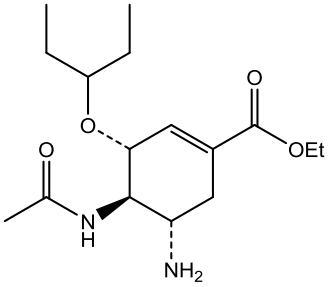


pentamidine

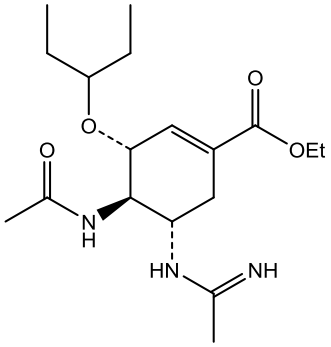


pentamidine-amidoxime: R = H
esters of pentamidine-amidoxime: R = acyl

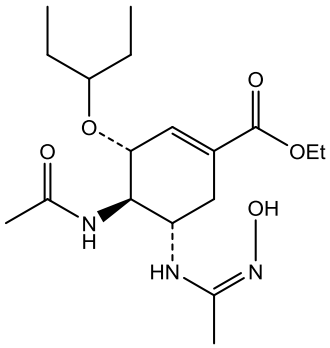
Hydroxylated amidines, guanidines and amidrazones have been patented as components for transdermal therapeutic systems [29, 30]. Orally available amidoxime prodrug has recently been prepared also for a derivative of **oseltamivir** [31].



oseltamivir



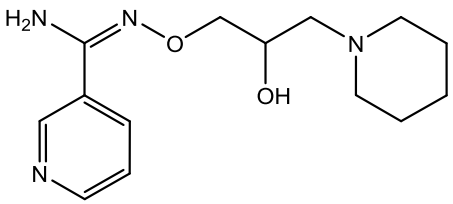
oseltamivir acetamidine analogue



amidoxime prodrug

Amidoxime have also been studied as potential NO (nitric oxide) donors since they liberate NO by nitric oxide synthase (NOS) independent mechanisms [32–36].

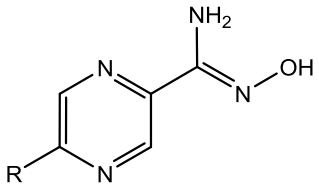
Amidoxime derivative **BGP-15** is an inhibitor of poly(ADP-ribose)polymerase and a heat shock proteins (HSP) inducer. It acts as a protective agent against side effects of other drugs [37–39] and has a positive effect on various conditions, such as neuronal injury and aging [40, 41], muscular dystrophy [42, 43], arthritis [44] and insulin resistance [45–49]. Currently, in undergoes clinical studies as a potential antidiabetic drug [49, 50]. BGP-15 and its analogues have also been patented as compounds that ameliorate the tissue regeneration effect of adult stem cells, their survival and adherence, and promote the regulation of adult stems cell differentiation [51].



BGP-15

2. Aim of the work

Research concerning amidoximes commenced at the Department of Pharmaceutical Chemistry and Pharmaceutical Analysis in 2011. At that time, Horká prepared a series of pyrazine-amidoximes (*N*′-hydroxy-5-alkylpyrazine-2-carboximidamide) within her diploma work [12].



General formula of pyrazine-amidoximes prepared by Horká

R = propyl, isopropyl, pentyl, hexyl

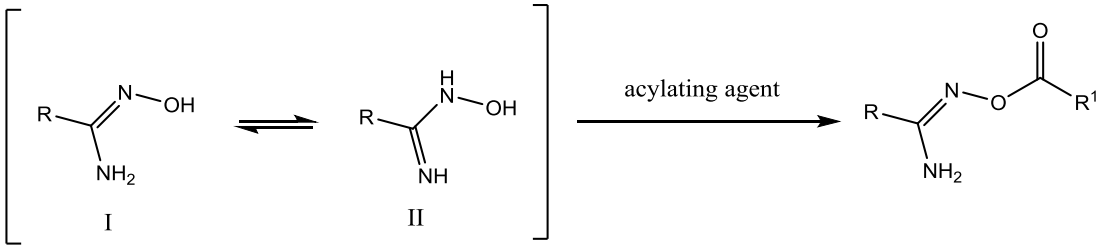
The compounds were tested for the antibacterial and antifungal activities. Although antibacterial effects of amidoximes have previously been reported [52–56], some heterocyclic amidoximes have been patented as agricultural fungicides [57, 58] and some amidoximes exhibited activity against *Aspergillus* spp. [56] the pyrazine-amidoximes were inactive in the assays performed at the Department of Biological and Medical Sciences. Therefore it was decided to prepare and test additional amidoximes and their derivatives.

Modifications of amidoxime group can yield *N*-substituted derivatives, *O*-substituted derivatives, *N*,*O*-disubstituted derivatives and cyclic analogues. My diploma work will be concerned with *O*-acylated amidoximes and their cyclization to 3,5-disubstituted-1,2,4-oxadiazoles.

3. Theoretical part

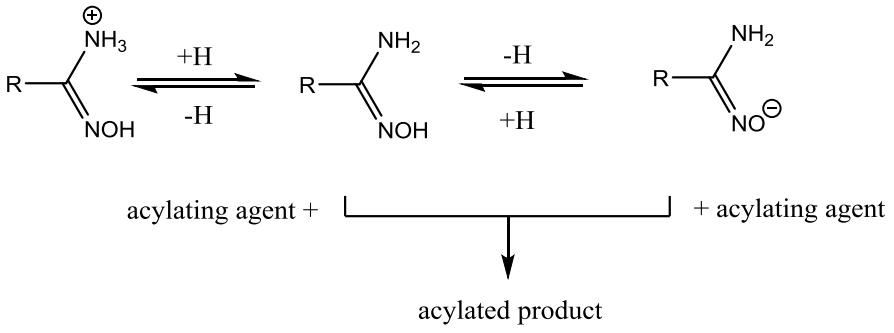
3.1 Acylation of amidoximes

Generally, acylation of amidoximes is done easily by anhydrides or acid chlorides [1]. It can also been performed using carboxylic acids activated by DCC, EDC, CDI, TBTU, HOBt or HBTU [59]. Special methods of acylation have also been described [2, 8, 11, 60].



Scheme 1 Acylation of amidoximes

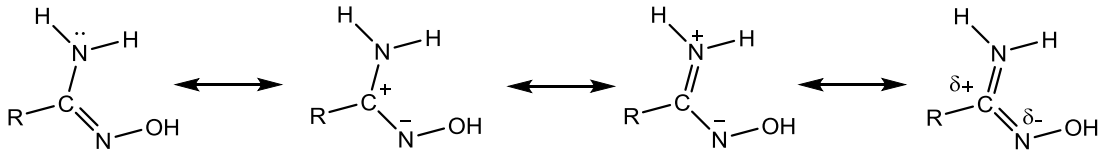
Amidoximes exist in two tautomers I and II, and it has been confirmed by IR and NMR spectra and other studies that the former tautomer is more stable [61–64]. They represent a special group of α-nuclephiles within the series of efficient acyl group acceptors – hydroxamic acids, oximes, and amidoximes. Although they are structurally similar to oximes, their kinetic behavior in reactions with the halides and esters of phosphorus and carboxylic acids differes substantially from kinetic behavior of oximes. In the case of oximes, the acceptor of the acyl group is the oximate anion. In amidoximes, both neutral and the anionic forms act as nucleophilic reagents [65].



Scheme 2 Acylation of amidoximes (adopted and modified from the ref. [65])

Irrespective of which form (neutral or anionic) reacts with the acylating reagent, the reaction product is always *O*-acylderivative [64, 65], except for formamidoxime where both *O*- and *N*-acylations occur [1, 64, 66].

Nucleophilicity of of nitrogen in NH_2 group is lower than that in normal NH_2 moiety due to mesomerism and formation of iminium cation C-NH_2 to C=NH_2^+. Thus electron density and nucleophilicity of OH- moiety is improved and acylation at this moiety takes place [67, 68].

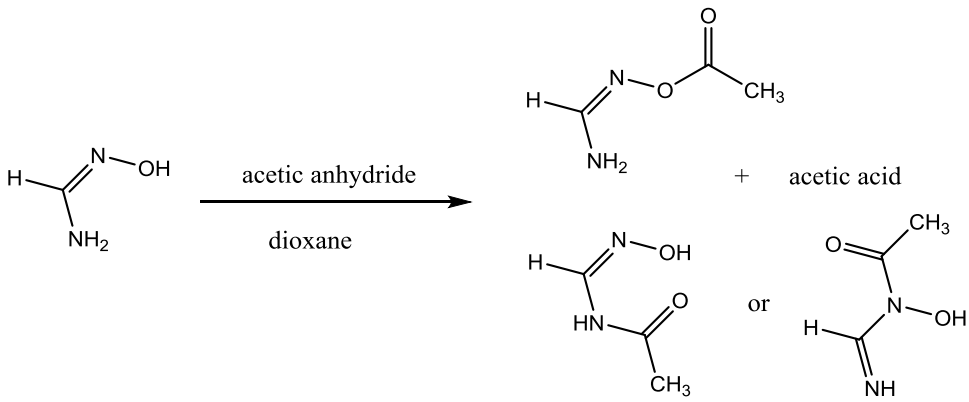


Scheme 3 Resonance forms of amidoximes (adopted and modified from ref. [69])

Acyl derivatives of amidoximes have basic character, no acidic like starting ones. This proves that isonitroso group is involved in acylation reaction. Additionally, the absence of OH absorption and the presence of NH_2 and –O-CO groups is obvious from IR spectra [1, 70].

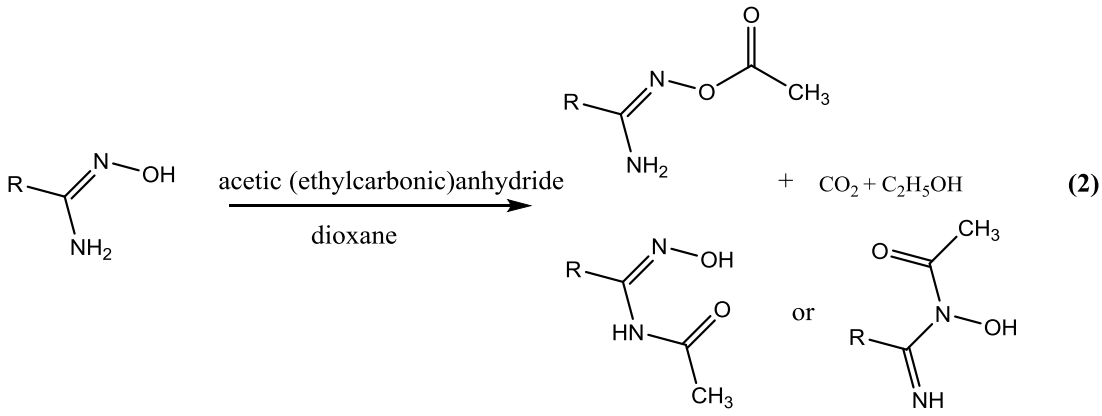
3.1.1 Acylation of amidoximes with anhydrides

Acylation of amidoximes with anhydrides can be limited by the lack of commercially available anhydrides [67]. Eloy and co-workers [66] performed acetylation of formamidoxime using acetic anhydride or mixed acetic (ethyl carbonic) anhydride. In both cases, they obtained both *O*-acetylated and *N*-acetylated products.



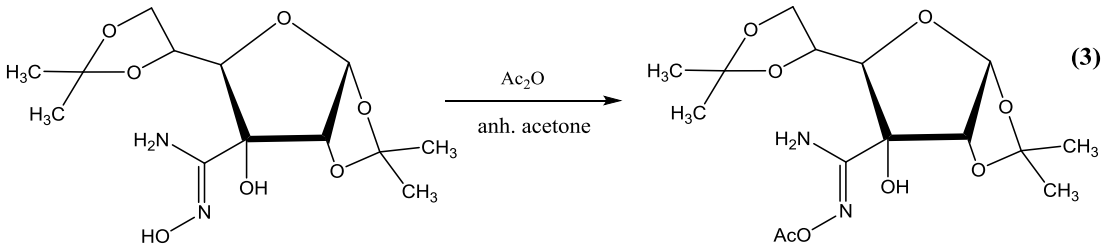
(1)

The advantage of the reaction with acetic (ethyl carbonic) anhydride is that no acetic acid, which complicates crystallization of the products, is formed [66].



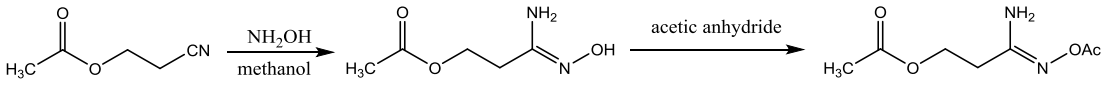
(2)

Acetylation of the amidoxime derived from a sugar was performed by acetic anhydride in anhydrous acetone [68].



(3)

Acetanhydride was also used to prepare *O*-acetylderivatives of acetylhydracrylamidoxime and ethoxycarbonylhydracrylamidoxime. It these cases acetic anhydride (without a solvent) was added to the crude amidoximes prepared from corresponding nitriles [70].



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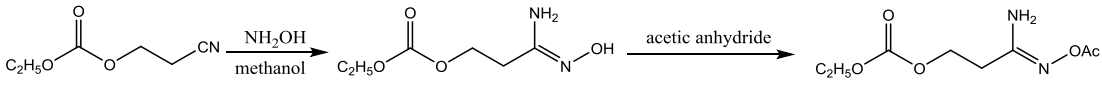
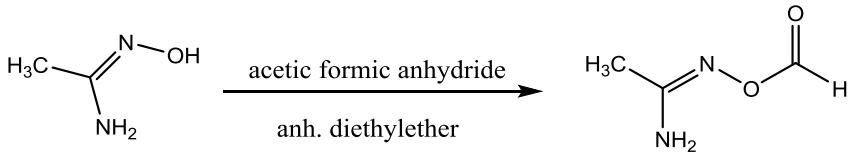
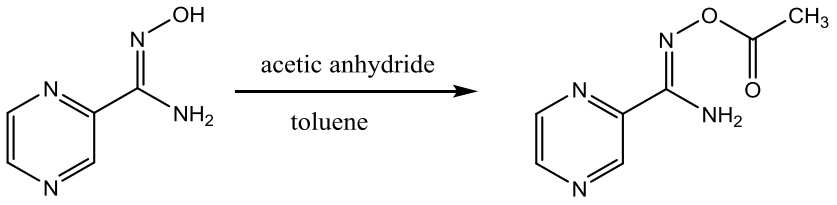


Formylation of acetamidoxime was performed by means of mixed acetic formic anhydride in dry diethylether [70].



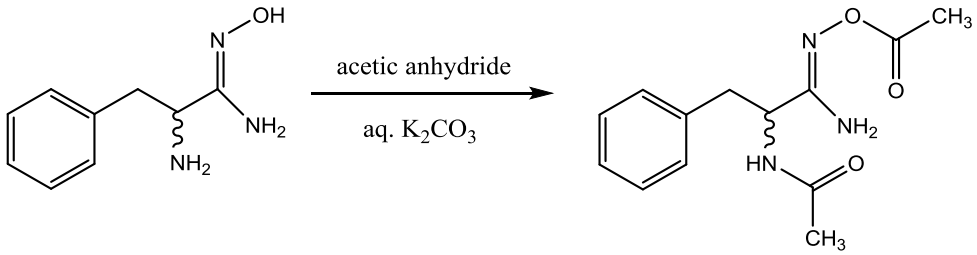
(5)

N′-Acetoxypyrazine-2-carboximidamide was obtained by heating *N*′-hydroxypyrazine-2-carboximidamide with acetic anhydride in toluene [71].



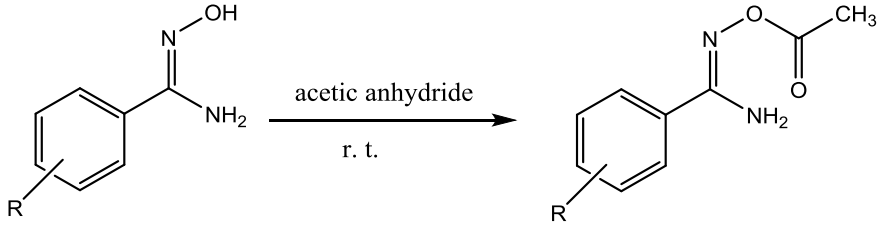
(6)

Heating of DL-2-amino-*N*′-hydroxy-3-phenylpropanimidamide with acetic anhydride and aqueous potassium carbonate yielded diacetylated product [72].



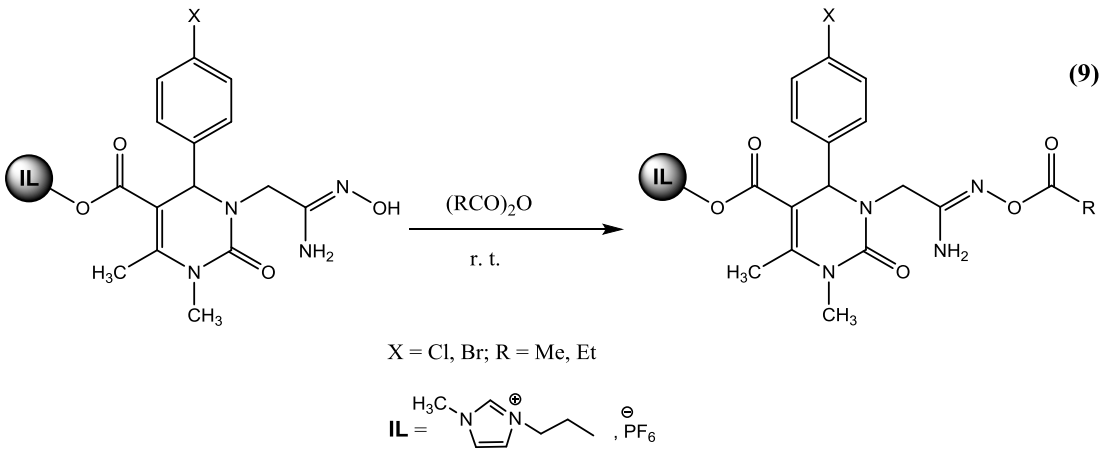
(7)

Benzamidoximes, where R = H, 4-N(CH_3)$_2$, 4-OCH_3, 4-Br, 3-Cl, 4-NO_2, gave acetylated derivatives when they were left to react with acetic anhydride at room temperature for 18 hours [73].



(8)

The same reaction conditions were use to prepare N-3 funtionalized 3,4-dihydropyrimidine-2(1*H*)-ones, where ionic liquid bound amidoxime reacted with acetic or propionic anhydride to yield the corresponding acylated product [74].



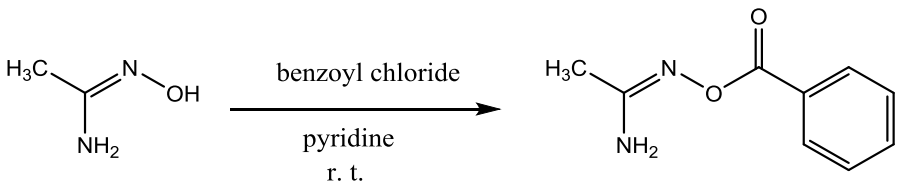
(9)

Anhydrides were applied also by other researchers [75–78].

3.1.2 Acylation of amidoximes with acyl chlorides

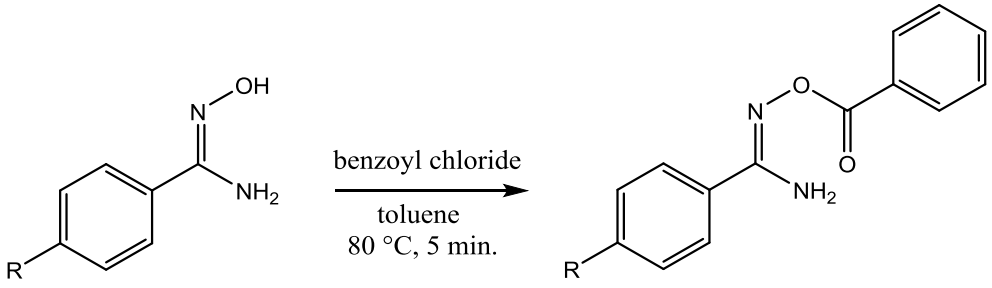
Acyl chlorides are very effective acylating agents and have been used for the preparation of both acylated [66, 67, 79, 80] and aroylated [61, 66, 67, 70, 72, 73, 75, 77, 81–84] amidoximes.

Bell and co-workers [61] prepared *O*-benzoylacetamidoxime using benzoyl chloride in pyridine at room temperature.



(10)

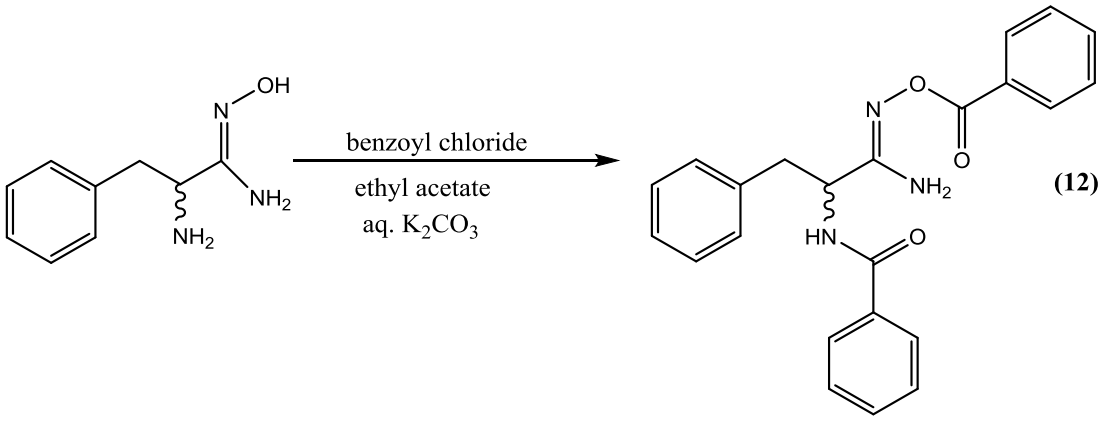
Benzoylchloride in pyridine was also used by Tsiulin *et al.* [82]. They performed the reaction at 80 °C. Equimolar amounts of the reactants were used. According to the IR data, the product was formed immediately after mixing solutions of *N*-hydroxybenzimidamide and benzoyl chloride in pyridine at 100°C, and the initial reactants are consumed completely. The benzoylated amidoximes were used to study reaction kinetic of their cyclization in different solvents.



(11)

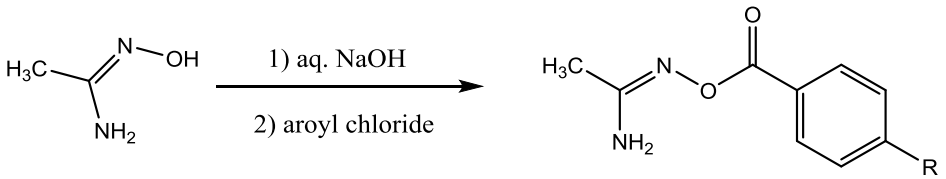
R = H, NO_2, Br, Me, MeO

When DL-2-amino-*N*′-hydroxy-3-phenylpropanimidamide in ethyl acetate was mixed with benzoyl chloride and aqueous potassium carbonate dibenzoylated derivative was formed immediately without heating [72].



(12)

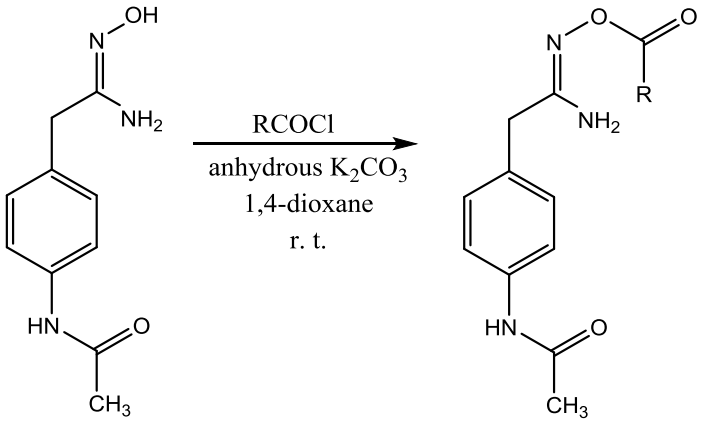
Ooi and Wilson [73] prepared a series of aroylated acetamidoximes by stirring acetamidoxime with aqueous sodium hydroxide. The solution was then filtered and treated with with the aroyl chloride, and the aroylated product was obtained. Similar procedure was used by Eloy et al. [70] to obtain benzoylated acetamidoxime.



(13)

R = H, 4-OCH_3, 3-F, 4-NO_2

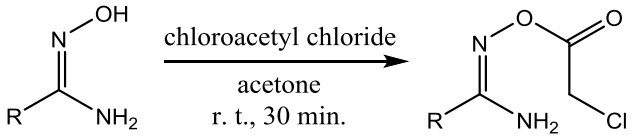
Reaction of *N*-{4-[2-amino-2-(hydroxyimino)ethyl]phenyl}acetamide with acyl- or aroyl chlorides after addition of anhydrous K_2CO_3 in 1,4-dioxane under room temperature leads to good yields of *O*-acyl amidoximes [79].



(14)

R= 1: methyl, 2: ethyl, 3: phenyl, 4: 3-pyridyl, 5: benzyl, 6: 2-phenylethyl, 7: 3-phenylprop-2-en-1-yl, 8: 3-(furan-2-yl)prop-2-en-1-yl, 9: *tert*-butyl

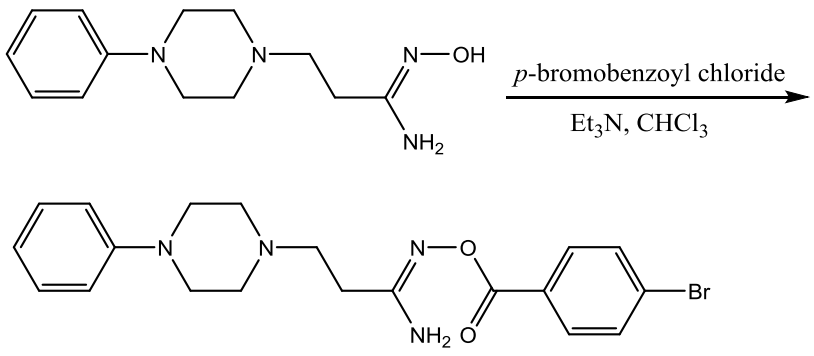
Chloroacetyl chloride acylates amidoximes in acetone at ambient temperature after 30 minutes [83].



(15)

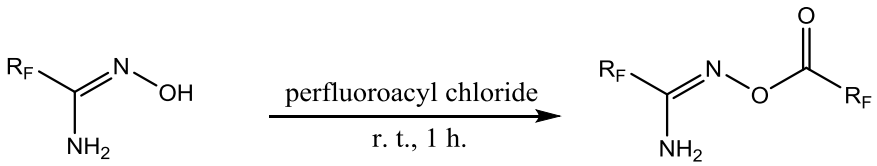
R= 1: C_6H_5, 2 : *p*-$CH_3C_6H_4$, 3 : *p*-$CF_3C_6H_4$,

One efficient method generating *O*-*p*-bromobenzoyl-β-(4-phenylpiperazine-1-yl) propioamidoximes is by adding *p*-bromobenzoyl chloride in $CHCl_3$ to a mixture of amidoxime with triethylamine (Et_3N) [84].



(16)

O-perfluoroacyl perfluoroacyl amidoximes were derived by treatment of perfluoroacyl amidoxime in anhydrous ether solution with perfluoroacyl chloride at room temperature for 1 hour [80].



(**17**)

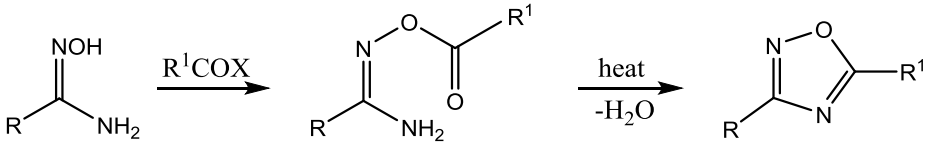
R_F= 1: CF_3, 2: C_2F_5, 3: C_3F_7

Acyl and aroyl chlorides were used as well by other researchers [66, 67, 70, 75, 77, 81].

3.2 Synthesis of 1,2,4-oxadiazoles

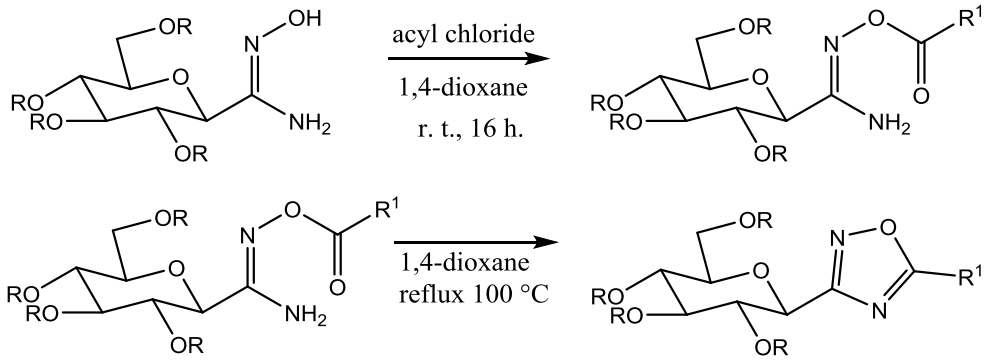
Oxadiazoles are on the focus of the research [1, 9, 67, 75, 76, 78–83, 85–89] because they are isosteres of esters and amides and this is the explanation supporting that they have similar biological properties with them. They can grant a big series of H_3-antagonists, monoamine oxidase inhibitors (MAOI), drugs against tumors or against inflammation, muscarinic antagonists, tyrosine kinase inhibitors [86]. Others showed antiparasitic, coronary artery dilating, myorelaxant, aldose reductase inhibiting and anesthetic activities [87]. Some oxadiazoles were tested as inhibitors of glycogen phosphorylase (GP) [88].

The so called "thermal cyclization" of *O*-acylamidoximes is the reaction which ends with 1,2,4-oxadiazoles [9].



Scheme 4 Preparation of 1,2,4-oxadiazoles

It is clear that oxadiazoles are formed in two step reaction, acylation and cyclization [67, 79–83]. The synthesis can be performed as a one-pot reaction without isolating the intermediate ester or in two steps. Thus, 5-substituted 3-*C*-β-D-glucopyranosyl-1,2,4-oxadiazoles (where R = benzyl) were prepared by heating the corresponding acylated amidoximes using a one-pot procedure, while in the case of 3-*C*-β-D-glucopyranosyl-1,2,4-oxadiazoles (where R = benzoyl) the intermediates were was first isolated and then cyclized under the same conditions [67].



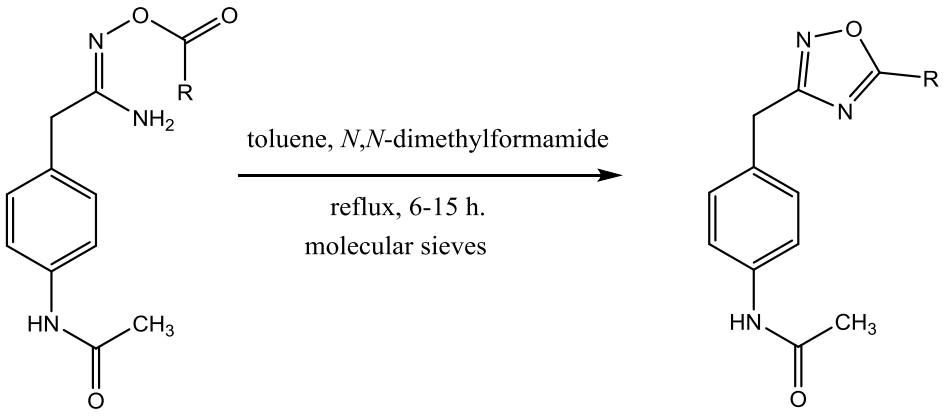
(18)

R = benzyl or benzoyl

R_1 = methyl, phenyl, 4-MeO-phenyl, 4-Me-phenyl, 4-NO_2-phenyl, 3-Cl-phenyl, 3-pyridyl, 2-furyl, 2-thienyl, 1-naphtyl, 2-naphtyl *etc.*

3.2.1 Procedures with the isolation of acylated intermediate

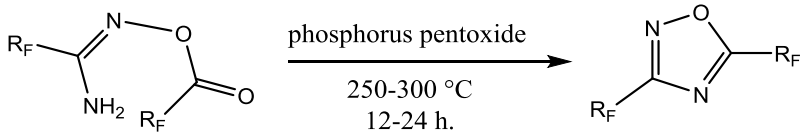
N-{4-[(5-substituted-1,2,4-oxadiazol-3-yl)methyl]phenyl}acetamides were generated in two step pathway too. Firstly, acylated amidoximes were prepared according to reaction **(14)**. Second step was submission of the intermediate to reflux in toluene and *N*,*N*-dimethylformamide in the presence of freshly dried molecular sieves (4 Å) for 6–15 hours [79].



(19)

R = 1: methyl, 2: ethyl, 3: phenyl, 4: 3-pyridyl, 5: benzyl, 6: 2-phenylethyl, 7: 3-phenylprop-2-en-1-yl, 8: 3-(furan-2-yl)prop-2-en-1-yl, 9: *tert*-butyl

3,5-bis(perfluoroalkyl)-1,2,4-oxadiazoles synthesis includes the treatment of *O*-perfluoroacyl perfluoroacyl amidoxime with phosphorus pentoxide at 250–300℃ for 12–24 hours [80]. Acylation step was mentioned in previous part **(17)**.

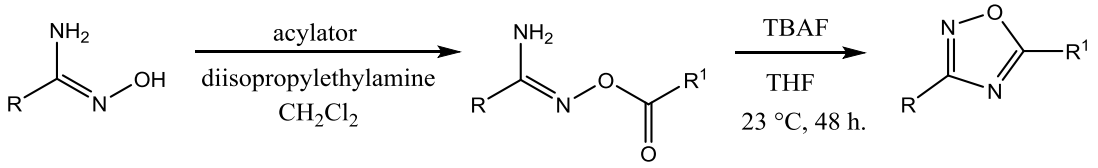


(20)

R_F = 1: CF_3, 2: C_2F_5, 3: C_3F_7

Various alkanoyl or aroyl amidoximes, prepared with a suitable acylator (acid anhydride or chloride) in dichloromethane in the presence of diisopropylethylamine, were further cyclized with tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) at 23 °C. It was found that a catalytic amount of TBAF could facilitate conversion of starting amidoximes and that reaction times were significantly shorter when 1.0 equivalent of TBAF was used. The reaction times were also dependent on the solvent used. Acetonitrile was comparable with THF, dichloromethane was less suitable. No oxadiazole formation was observed in the absence of TBAF, only trifluoromethyl benzamidoxime (R = phenyl, R^1 = CF_3) cyclized spontaneously after treatment with trifluoroacetic anhydride [81].

(21)

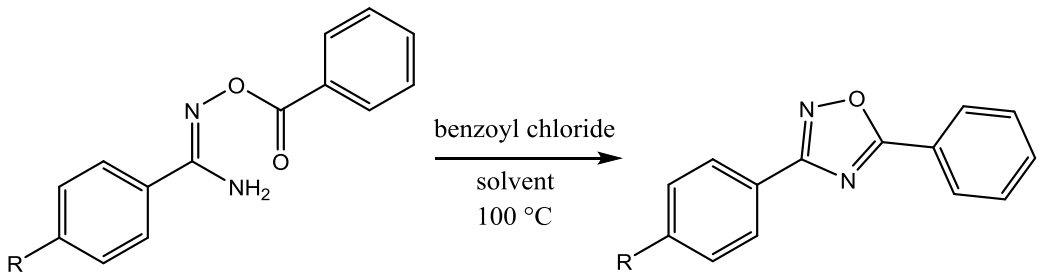


R = methyl, phenyl or subst. phenyl

R^1 = methyl, methoxy, *tert*-butyl, chloromethyl, 2-oxopropyl, methoxycarbonylmethyl, trifluoromethyl, isopropyl, phenyl, 2-nitrophenyl, 3-nitrophenyl, 4-nitrophenyl, benzyl

Benzoylated benzamidoximes synthesized according to reaction **(11)** were further cyclized to corresponding 3,5-disubstituted-1,2,4-oxadiazoles [82].

(22)



R = H, NO_2, Br, Me, MeO

The cyclization was performed in the following solvents: glacial acetic acid, pyridine, 1,4-dioxane and toluene. Equimolar amounts of the reagents were used and the reaction mixture was heated to 100 °C. To study reaction kinetic, benzoyl chloride was quickly added to the benzoylated amidoxime and samples of the reaction mixture were taken during the process and diluted with 15–20 volumes of acetonitrile. The reaction rate was determined from the accumulation of final product which was quantitated by HPLC [82].

It was found that the cyclization can be described by first-order kinetic equation. It is favored by electron-donating substituents, and is dependent on reaction medium as shown in Fig. 1.

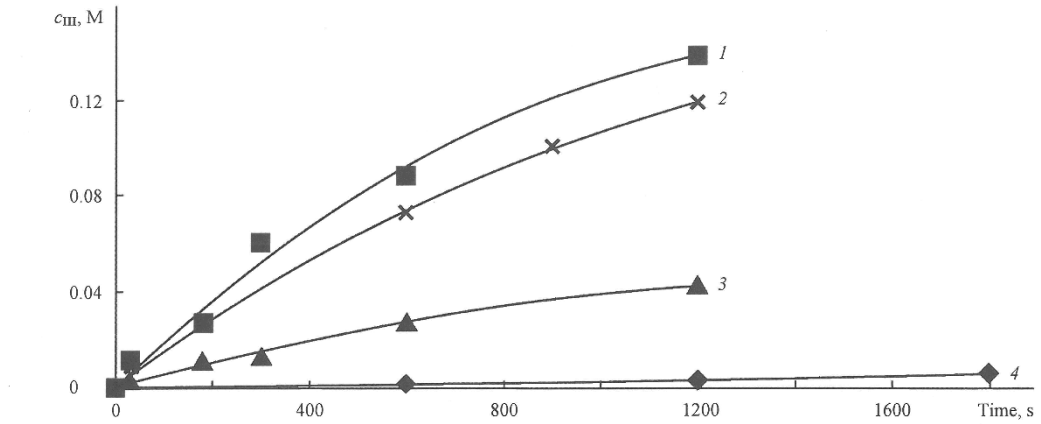
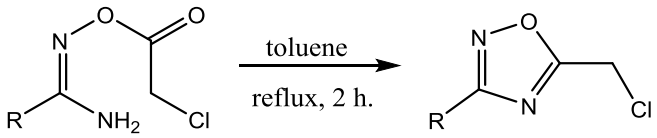


Fig. 1 Cyclization of *N′*-(benzoyloxy)benzimidamide in (*1*) glacial acetic acid, (*2*) pyridine, (*3*) 1,4-dioxane, and (*4*) toluene; c_{III} is the concentration of the oxadiazole (adopted from ref. 82)

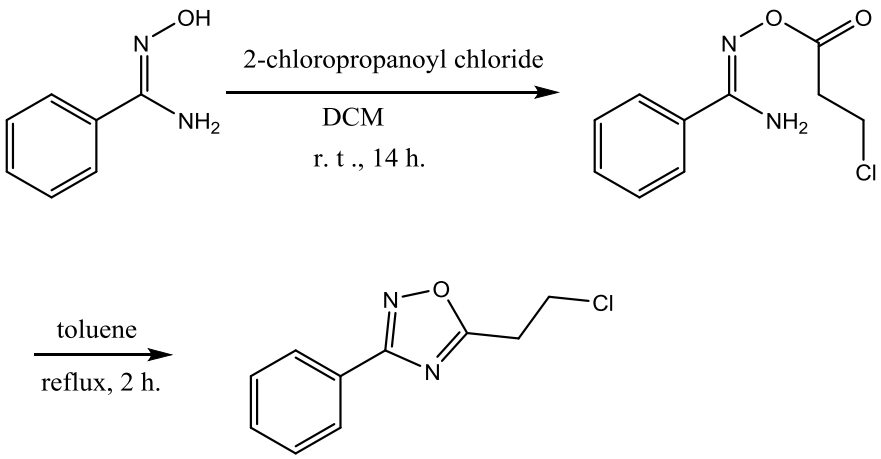
Chloroacetyl chloride reacts with amidoxime in acetone at room temperature for 30 minutes as shown in reaction **(15).** *O*-chloroacetyl derivative produced is put under reflux in toluene for 2 hours and gives 5-chloromethylated 1,2,4-oxadiazole [83].



(23)

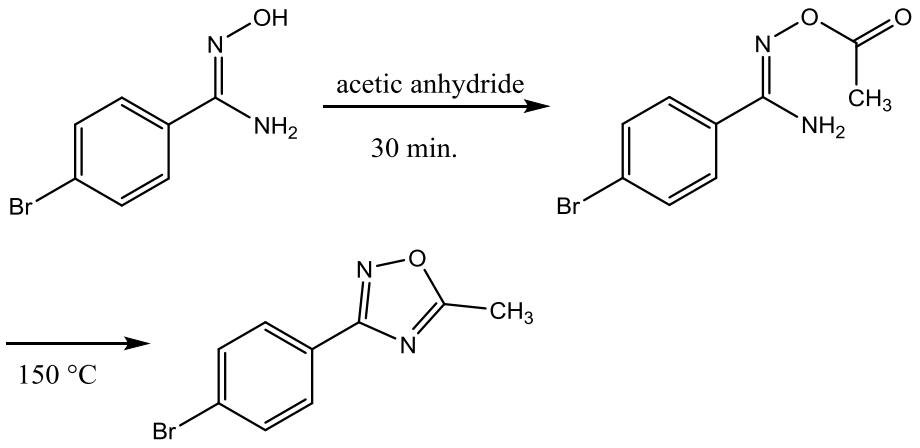
R = 1: C_6H_5, 2: *p*-$CH_3C_6H_4$, 3: *p*-$CF_3C_6H_4$

In a similar way benzamidoxime acylated with 2-chloropropanoyl chloride in dichloromethane gives 5-(2-chloroethyl)-3-phenyl-1,2,4-oxadiazole [83].



(24)

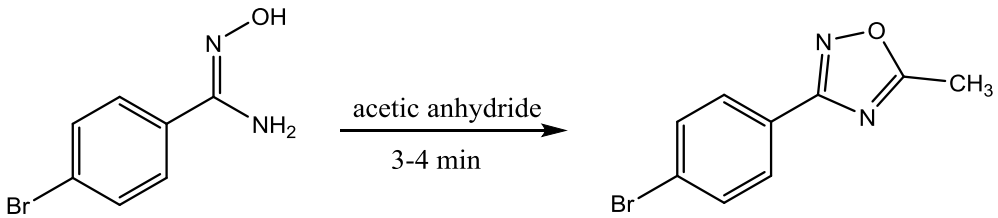
Cyclization can also be performed in a solvent-free manner. For instance, *O*-acetyl-4-bromobenzamidoxime prepared by acetylation of the corresponding amidoxime leads to 3-(4-bromophenyl)-5-methyl-1,2,4-oxadiazole when heated over its melting point (145 °C) [75].



(25)

3.2.2 One-pot procedures without the isolation of acylated intermediate

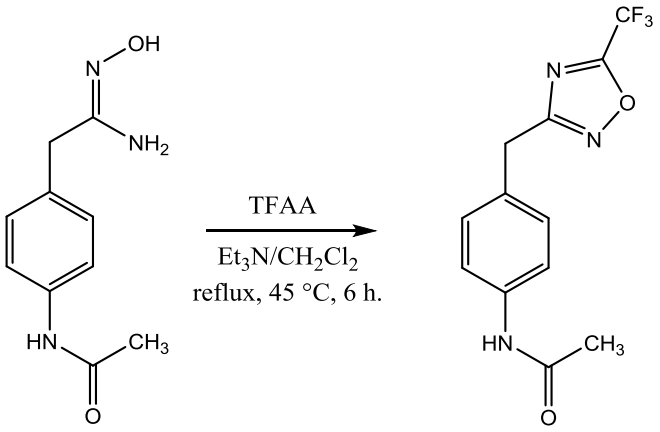
The latter compound was also prepared using one-pot procedure in which 4-bromobenzamidoxime was heated with acetic anhydride for 3–4 minutes. One-pot procedure gave a slightly higher yield that the two step reaction [75].



(26)

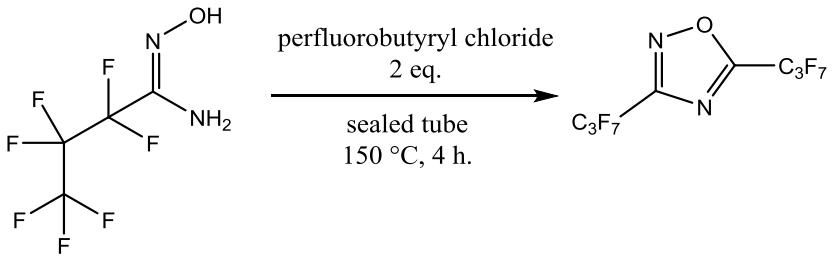
One-pot procedures were also used by other researchers [79, 80, 86–89].

To prepare *N*-[4-(5-trifluoromethyl-1,2,4-oxadiazol-3-yl)methyl)phenyl]acetamide the authors [79] used one-pot reaction of starting amidoxime with trifluoroacetic anhydride (TFAA) in a mixture of anhydrous dichloromethane (CH_2Cl_2) and triethylamine (Et_3N) at 45 C for 6 hours.



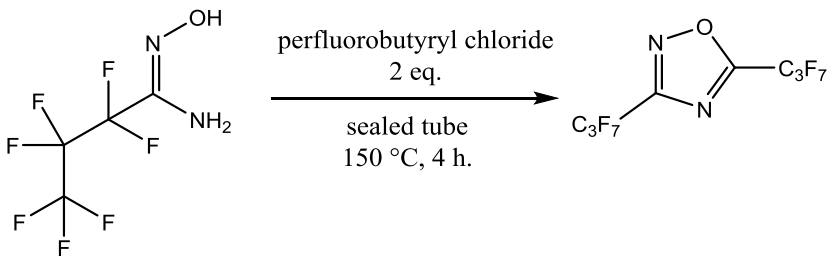
(27)

For the synthesis of 3,5-bis(perfluoroalkyl)-1,2,4-oxadiazoles two modifications of the method depicted in reaction **(20)** were found in which acylation and dehydratation were achieved in one step. In the first one, perfluorobytyryl chloride functioned as both acylating and dehydrating agent. The reaction was performed in a sealed tube at 150 °C for 4 hours [80].



(28)

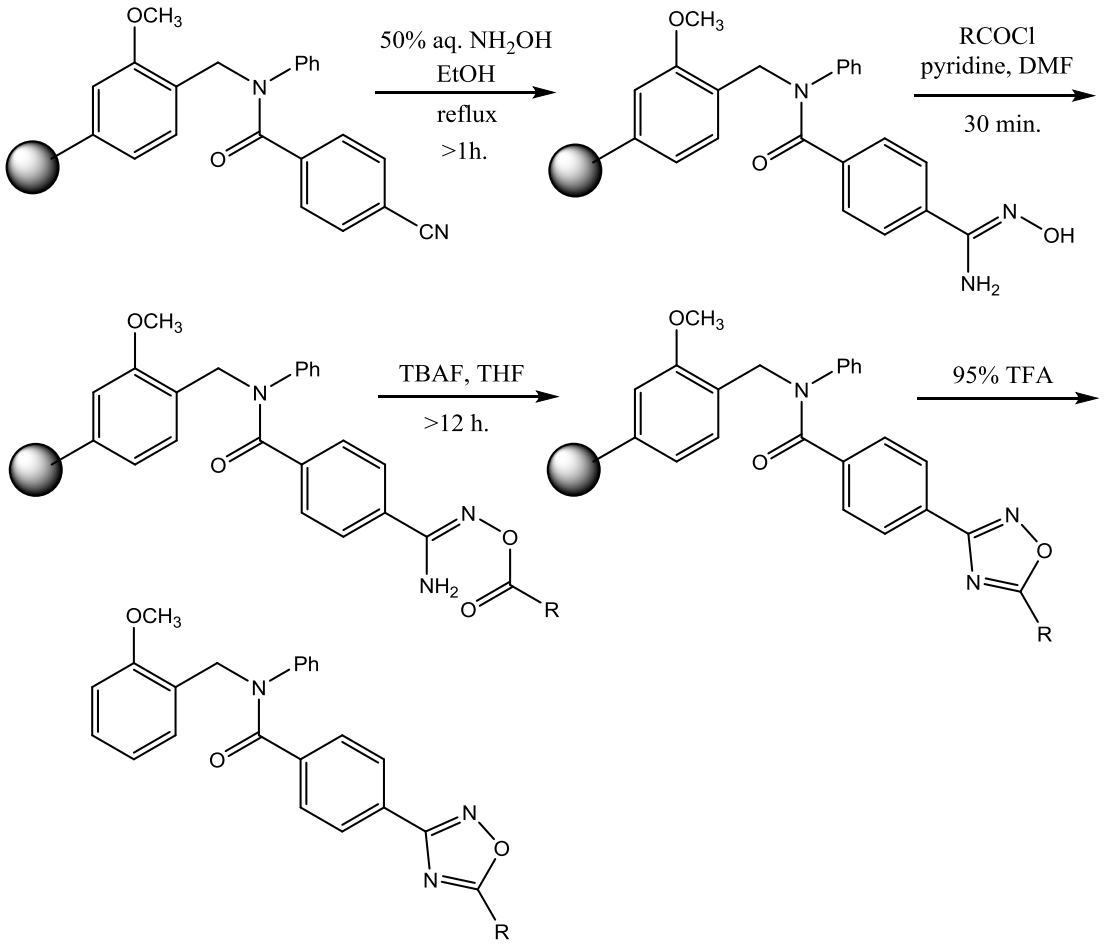
In the second modification, perfluorobutyramidoxime was mixed with phosphorus pentoxide and perfluorobutyric acid. The reaction mixture was then refluxed for 6 hours. The reaction probably involves initial formation of perfluorobutyric anhydride, which acylates the perfluorobutyramidoxime, and, with phosphorus pentoxide, also acts as a dehydrating agent [80].



(28)

Rice and Nuss [86] performed synthesis of 3,5-disubstituted-1,2,4-oxadiazoles on the solid support using tetra-*N*-butylammonium fluoride (TBAF) for the cyclodehydration of *O*-acylamidoximes. Nitriles bound to Agropore MB-CHO resin were converted to amidoximes using a range of acid chlorides. Subsequent treatment with TBAF in THF under ambient conditions gave a library of 3,5-disubstituted-1,2,4-oxadiazoles. All reactions steps were performed in a one-pot manner [86].

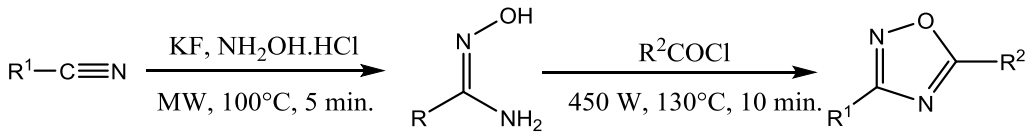
(30)



R = methyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, *tert*-butyl, phenyl, phenylacetyl, hydrocinnamyl, subst. phenyl, mesityl, 4-biphenyl, 1-naphtyl, 2-naphtyl, 2-furyl, thiofen-2-yl, 4-pyridyl, 3-pyridyl, 3,5-dimethyloxazol-2-yl, 1-phenyl-5-propylpyrazol-4-yl

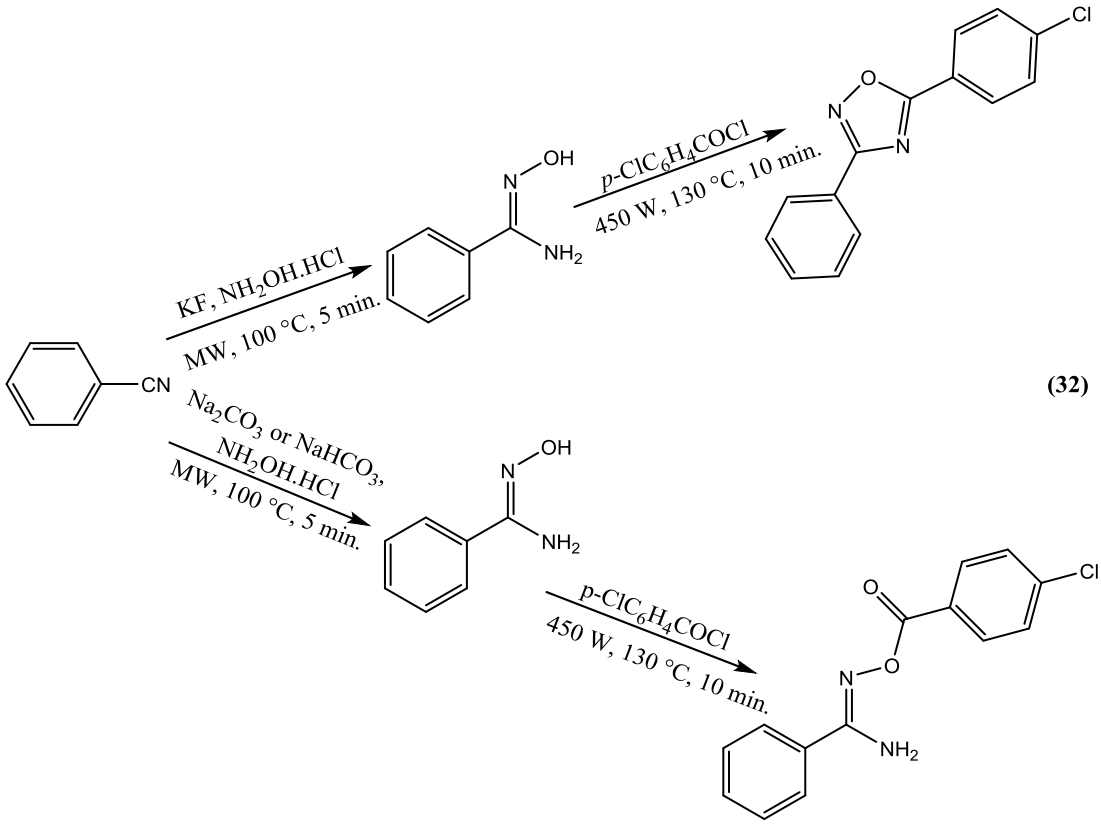
A profitable and more enviromentally friendly method for the preparation of 3,5-disubstituted-1,2,4-oxadiazoles could be working without a solvent, aided by microwave irradiation, catalyzed and solid supported by potassium fluoride. It is a one-pot synthesis starting from nitriles [87].

(31)



R^1 = phenyl, subst. phenyl, benzyl
R^2 = phenyl, subst, phenyl

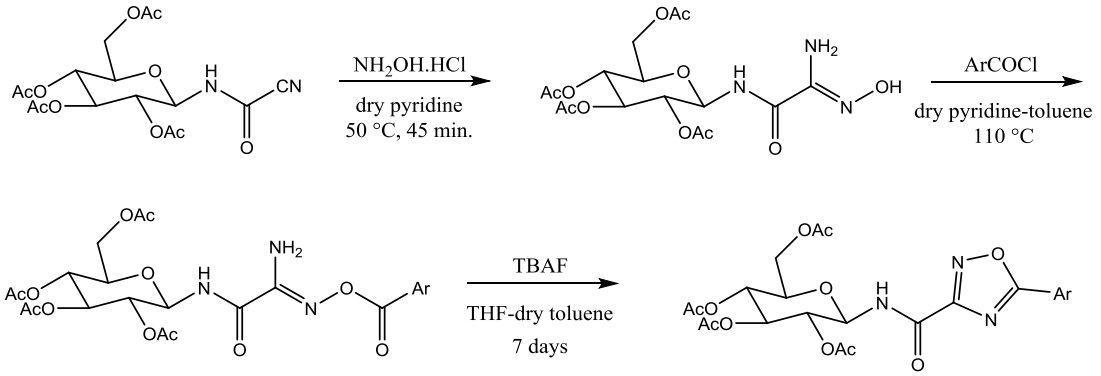
In order to assess the ability of potassium fluoride to catalyze the reaction, sodium carbonate and sodium bicarbonate were examined also, using *p*-chlorobenzoyl chloride as acylating agent. In the later cases the dominant outcome was *O*-acyl derivative [87].



(32)

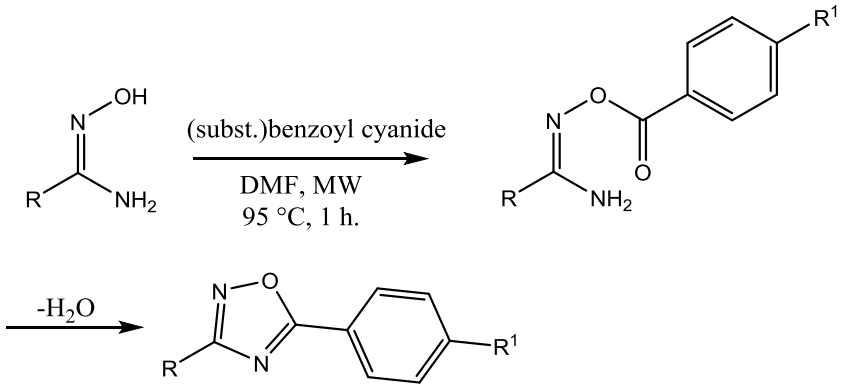
Polyák and co-workes [88] prepared 5-aryl-*N*-(2,3,4,6-*O*-acetyl-β-D-glucopyranosyl)-1,2,4-oxadiazole-3-carboxamides in a one-pot procedure using TBAF as the cyclizing agent.

(33)



Ar = phenyl, 2-naphtyl, 1-naphtyl

3,5-disubstituted-1,2,4-oxadiazoles were also obtained by reacting benzamidoximes with benzoyl cyanides in various solvents (1,4-dioxane, toluene, DMF, acetonitrile) at different temperatures under both conventional as well as microwave heating conditions. It was found that microwave irradiation decreased the reaction time and increased the yields of the target compounds. DMF proved to be the most convenient solvent [89]. The authors also stated that the cyclization was promoted by electron-donating substituents on the amidoxime benzene ring, which in agreement with the conclusions of Tsiulin and co-workers [82]. On contrary, electron-withdrawing substituent on benzoyl cyanide benzene ring is favorable for the cyclization [89].

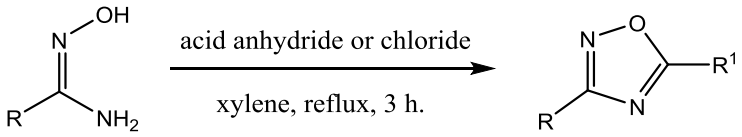


(34)

R = phenyl, 2-OCH_3-phenyl, 3-OCH_3-phenyl, 4-OCH_3-phenyl, 2-NO_2-phenyl, 3-NO_2-phenyl, 4-NO_2-phenyl, cyclohexyl, adamantyl

R^1 = H, F, CH_3

One-pot procedure was also used by Fanshawe and Safir [90–92] who prepared 3,5-disubstituted-1,2,4-oxadiazoles by heating amidoximes with an acid anhydrides or chlorides in xylene.



(35)

R = cyclopropyl, 4-pyridazinyl, 4-pyrimidinyl, 2-pyrazinyl,
R^1 = methyl, ethyl, cyclopropyl,

4. Experimental part

Commercially available substances were used for the preparation for the substances prepared in this thesis:

- pyrazinecarbonitrile, 99% (Aldrich)
- hydroxylamine hydrochloride, p.a. (Lachema)
- acetic anhydride, pure (Penta)
- trimethylacetic anhydride, 99% (Aldrich)
- 2,3-pyrazinedicarboxylic anhydride, 97% (Aldrich)

TLC was performed on TLC aluminium sheets, silica gel 60 F_{254} (Merck). Light petroleum + ethyl acetate 60:40 (v/v) and toluene: acetone 70:30 were used as a mobile phases.

For analysis, the samples of compounds were dried 24 hours in the dessicator over anhydrous phosphorus pentoxide at 1.33 kPa and room temperature.

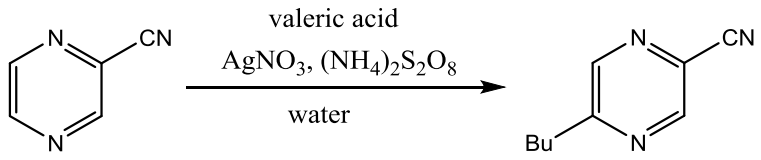
Melting points were determined using Melting Point Apparatus SPM 20 (Stuart) and are uncorrected.

Elemental analyses were performed with the EA 1110 CHNS Analyzer (Carlo Erba).

IR spectra were recorded with the spectrophotometer NICOLET 6700 using ATR-Ge method. Wavenumbers are given in cm^{-1}.

^{1}H NMR a ^{13}C NMR spectra were recorded with the VARIAN Mercury-VxBB 300 spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C or VNMR S500 spectrometer operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR). Chemical shifts were recorded as δ values in ppm and were indirectly referenced to tetramethylsilane (TMS) via the solvent signal (2.49 for ^{1}H and 39.7 for ^{13}C in DMSO. Coupling constants (*J*) are given in Hz.

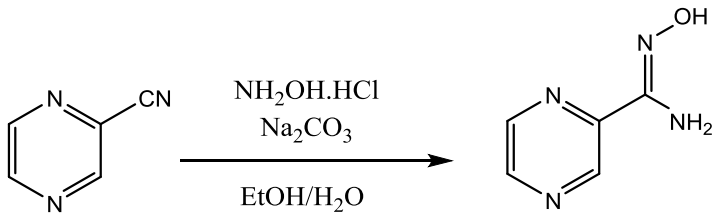
4.1 5-butylpyrazine-2-carbonitrile



To a solution of pyrazinecarbonitrile (10.5 g, 0.1 mol) in water (300 ml) heated to 80 °C, silver nitrate (1.7 g, 0.01 mol) and the valeric acid (10.2 g, 0.1 mol) were added. Ammonium peroxydisulfate (25.1 g, 0.11 mol) in water (70 ml) was then added dropwise whilst stirring and temperature maintained between 75–80 °C. The reaction mixture was stirred for 1 h. After cooling, the pH was adjusted to 9 with 10% solution of sodium hydroxide, and the mixture was continuously extracted with diethyl ether. The organic extract was dried over anhydrous sodium sulfate and filtrated.

The filtrate was adsorbed to 150 g Silica gel 60 Fluka, 0.063–0.200 mm and subjected to column chromatography using light petroleum + ethyl acetate 80:20 the mobile phase. Repeated chromatography yielded 6.2 g (40%) of a yellowish liquid, TLC of which corresponded to that of standard [93].

4.2 *N′* -hydroxypyrazine-2-carboximidamide



Pyrazine-2-carbonitrile (6.3 g; 0.06 mol) was diluted with 9 ml of ethanol. Hydroxylamine hydrochloride (8.34 g; 0.12 mol) was dissolved in 9 ml of water, and the solution was added to the ethanolic solution of the nitrile. Sodium carbonate (12.72 g; 0.12 mol) was dissolved in 75 ml of water and added cautiously to the reaction mixture. The mixture was then heated under reflux condenser at 70 °C for 5 hours and left standing at room temperature overnight. The crystals were separated through a sintered glass and washed with water. 7.82 g (94%) of the crude product was obtained. Crystallization from anhydrous ethanol gave 6.64 g (80%) of white needles.

Molecular weight: 138.13 ($C_5H_6N_4O$)

Melting point: 185–187 °C (ref. [53]: 186–187 °C, methanol; ref. [94]: 185–186 °C, sublimation)

Elemental analysis:

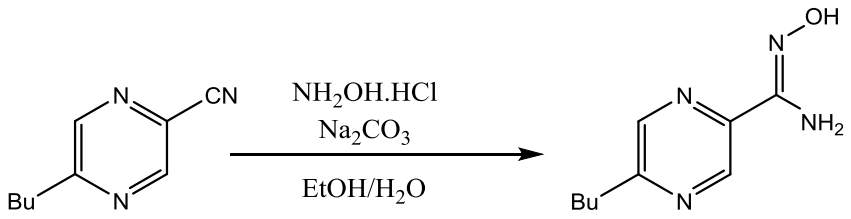
	% C	**% H**	**% N**
Calculated:	43.48	4.38	40.56
Found:	43.62	4.64	40.36

IR spectrum: 3434, 3300 (assoc. NH), 3147 (assoc. OH), CH, (pyrazine), 1659 (C=N), 953 (N-O)

^{1}H NMR spectrum (300 MHz, DMSO) δ 10.23 (1H, s, OH), 9.05 (1H, d, *J* = 1.4, H3), 8.64–8.59 (2H, m, H5, H6), 5.95 (2H, bs, NH_2)

^{13}C NMR spectrum (75 MHz, DMSO) δ 148.5, 146.0, 144.5, 143.3, 141.8

4.3 5-butyl-N′-hydroxypyrazine-2-carboximidamide



5-Butylpyrazine-2-carbonitrile (6.20 g; 0.038 mol) was diluted with 5.7 ml of ethanol. Hydroxylamine hydrochloride (5.28 g; 0.076 mol) was dissolved in 5.7 ml of water, and the solution was added to the ethanolic solution of pyrazine-2-carbonitrile. Sodium carbonate (8.06 g; 0.081 mol) was dissolved in 47.5 ml of water and added cautiously to the reaction mixture. The mixture was then heated under reflux condenser at 70 °C for 5 hours. After cooling, the crystals were separated through a sintered glass and washed with water. 5.9 g (79%) of the crude product was obtained. Crystallization from anhydrous ethanol gave 3.89 (52%) of colorless needles.

Molecular weight: 194.24 ($C_9H_{14}N_4O$)

Melting point: 154–155 °C

Elemental analysis:

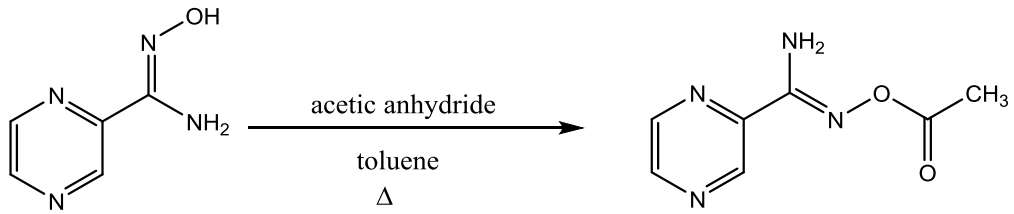
	% C	**% H**	**% N**
Calculated:	55.65	7.27	28.85
Found:	55.44	7.01	28.20

IR spectrum: 3431, (assoc. NH), 3179 (assoc. OH), 2955, 2928, 2862 (CH, aliph.), 1661 (C=N), 945 (N-O)

^{1}H NMR spectrum (500 MHz, DMSO) δ 10.11 (1H, s, OH), 8.96 (1H, s, H3), 8.50 (1H, s, H6), 5.90 (2H, bs, NH_2), 2.78 (2H, t, J = 7.6, CH_2), 1.71–1.61 (2H, m, CH_2), 1.36–1.26 (2H, m, CH_2), 0.89 (3H, t, *J* = 7.6, CH_3)

^{13}C NMR spectrum (125 MHz, DMSO) δ 157.0, 148.5, 143.4, 142.3, 140.8, 34.1, 31.0, 21.9, 13.9

4.4 *N′*-acetoxypyrazine-2-carboximidamide



N′-Hydroxypyrazine-2-carboximidamide (0.69 g; 0.005 mol) was suspended in toluene (100 ml). Acetic anhydride (0.51 g; 0.005 mol) was added, and the mixture was refluxed for 1 hour. Toluene was removed in vacuum and 0.73 g (81%) of the crude product was obtained. Crystallization from anhydrous ethanol gave 0.66 g (73%) of colorless needles.

Molecular weight: 180.17 ($C_7H_8N_4O_2$)

Melting point: 152–153 °C (ref. [71]: 168–169 °C, benzene)

Elemental analysis:

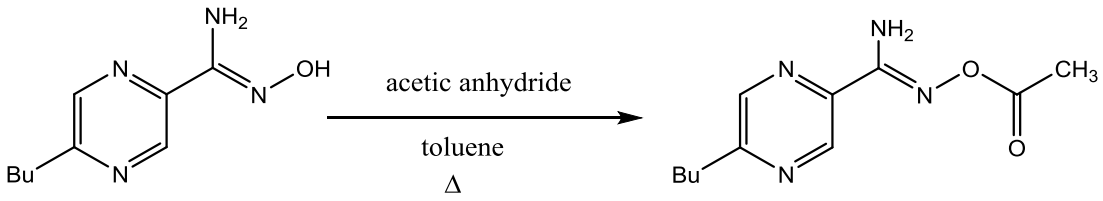
	% C	**% H**	**% N**
Calculated:	46.67	4.48	31.10
Found:	46.85	4.71	31.23

IR spectrum: 3436, 3352 (assoc. NH), 1744 (C=O), 1624 (C=N)

^{1}H NMR spectrum (300 MHz, DMSO) δ 9.10 (1H, d, *J* = 1.4, H3), 8.78 (1H, d, *J* = 2.5, H6), 8.73 (1H, dd, *J* = 2.5, *J* = 1.4, H5), 7.08 (2H, bs, NH_2), 2.17 (3H, s, CH_3)

^{13}C NMR spectrum (75 MHz, DMSO) δ 168.4, 153.2, 146.4, 144.5, 143.8, 142.7, 19.9

4.5 *N′*-acetoxy-5-butylpyrazine-2-carboximidamide



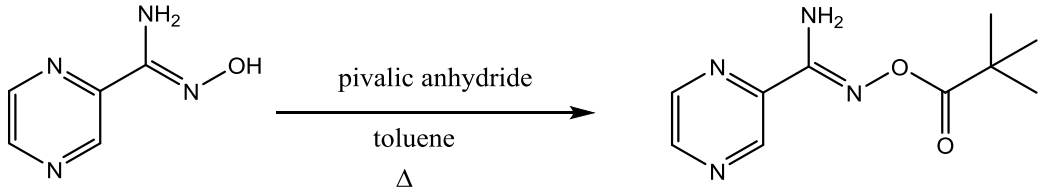
5-Butyl-*N′*-hydroxypyrazine-2-carboximidamide (0.97 g; 0.005 mol) was suspended in toluene (100 ml). Acetic anhydride (0.51 g; 0.005 mol) was added, and the mixture was refluxed for 1 hour. Toluene was removed in vacuum and 1.10 g (93%) of the crude product was obtained. Crystallization from anhydrous ethanol gave 0.91 g (77%) of colorless flakes.

Molecular weight: 236.27 ($C_{10}H_{13}N_4O_2$)
Melting point: 130–132 °C
Elemental analysis:

	% C	**% H**	**% N**
Calculated:	55.92	6.83	23.71
Found:	55.72	6.27	23.39

IR spectrum: 3428, 3315 (assoc. NH), 2962, 2930, 2859 (CH, aliph.), 1762 (C=O), 1632 (C=N)
^{1}H NMR spectrum (300 MHz, DMSO) δ 8.97 (1H, d, *J* = 1.5, H3), 8.60 (1H, d, *J* = 1.5, H6), 7.00 (2H, bs, NH_2), 2.82 (2H, t, *J* = 7.6, CH_2), 2.15 (3H, s, CH_3), 1.73–1.58 (2H, m, CH_2), 1.38–1.21 (2H, m, CH_2), 0.88 (3H, t, *J* = 7.6 , CH_3)
^{13}C NMR spectrum (75 MHz, DMSO) δ 168.4, 159.2, 153.4, 143.0, 141.9, 141.7, 34.3, 31.0, 21.9, 19.9

4.6 *N′*-pivaloyloxypyrazine-2-carboximidamide



N′-Hydroxypyrazine-2-carboximidamide (0.69 g; 0.005 mol) was suspended in toluene (100 ml). Pivalic anhydride (0.93 g; 0.005 mol) was added, and the mixture was refluxed for 1 hour. Toluene was removed in vacuum and 1.10 g (97%) of the crude product was obtained. Crystallization from anhydrous ethanol gave 0.75 g (66%) of colorless needles.

Molecular weight: 222.25 ($C_{10}H_{14}N_4O_2$)

Melting point: 156–157 °C

Elemental analysis:

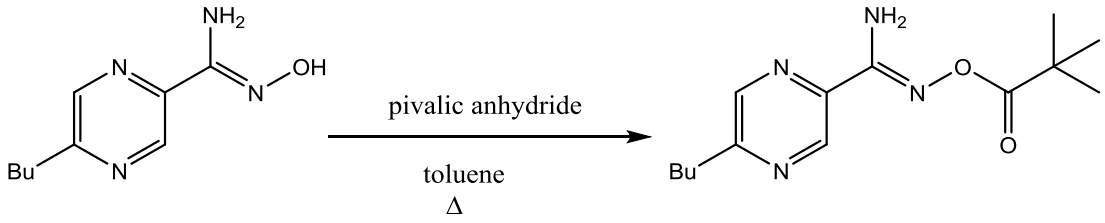
	% C	**% H**	**% N**
Calculated:	54.04	6.35	25.21
Found:	54.53	6.60	25.75

IR spectrum: 3498, 3382 (assoc. NH), 2981, 2937 (CH, aliph.), 1743, 1736 (C=O), 1625 (C=N)

^{1}H NMR spectrum (300 MHz, DMSO) δ 9.09 (1H, d, *J* = 1.2, H3), 8.78 (1H, d, *J* = 2.4, H6), 8.74–8.71 (1H, m, H5), 6.89 (2H, bs, NH_2), 1.25 (9H, s, CH_3)

^{13}C NMR (75 MHz, DMSO) δ 174.6, 153.0, 146.4, 144.6, 143.8, 142.8, 38.5, 27.2

4.7 5-butyl- *N′*-pivaloyloxypyrazine-2-carboximidamide



5-Butyl-*N′*-hydroxypyrazine-2-carboximidamide (0.97 g; 0.005 mol) was suspended in toluene (100 ml). Pivalic anhydride (0.93 g; 0.005 mol) was added, and the mixture was refluxed for 1 hour. Toluene was removed in vacuum and 0.87 g (63%) of the crude product was obtained. Crystallization from anhydrous ethanol gave 0.73 g (53%) of colorless needles.

Molecular weight: 278.36 ($C_{14}H_{22}N_4O_2$)

Melting point: 158–159 °C

Elemental analysis:

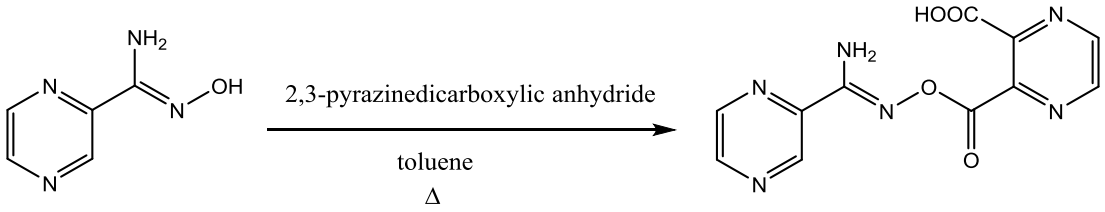
	% C	**% H**	**% N**
Calculated:	60.41	7.97	20.13
Found:	60.89	8.25	20.68

IR spectrum: 3400, 3296 (assoc. NH), 2963, 2930, 2872 (CH, aliph.), 1746 (C=O), 1639 (C=N)

^{1}H NMR spectrum (300 MHz, DMSO) δ 8.97 (1H, d, *J* = 1.5, H3), 8.62 (1H, d, *J* = 1.5, H6), 6.82 (2H, bs, NH_2), 2.83 (2H, t, *J* = 7.5, CH_2), 1.75–1.58 (2H, m, CH_2), 1.39–1.22 (2H, m, CH_2), 1.25 (9H, s, CH_3), 0.89 (3H, t, *J* = 7.5, CH_3)

^{13}C NMR spectrum (75 MHz, DMSO) δ 174.9, 159.1, 153.2, 143.0, 142.0, 141.7, 38.5, 34.3, 31.0, 27.3, 22.0, 13.9

4.8 3-[({[amino(pyrazin-2-yl)methylidene]amino}oxy)carbonyl] pyrazine-2-carboxylic acid



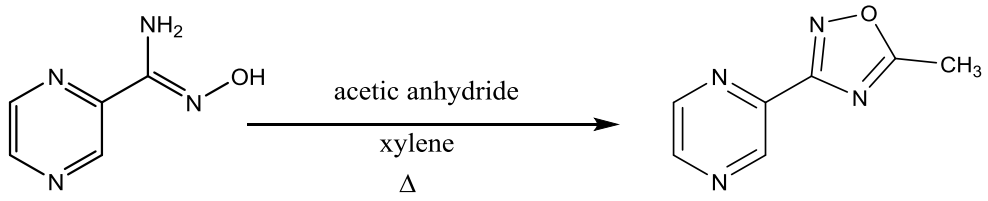
N′-Hydroxypyrazine-2-carboximidamide (0.69 g; 0.005 mol) was suspended in toluene (100 ml) and heated until the amidoxime dissolved. Anhydride of 2,3-pyrazinedicarboxylic acid (0.75 g; 0.005 mol) was then added. A solid started to precipitate immediately. The mixture was refluxed for 1 hour (the precipitate did not dissolve during heating). After cooling, the solid was filtered off, washed with a small of toluene and a small amount of ethanol. After drying, 1.05 g of an off-white powder was obtained. According to TLC results, this substance was a mixture of two compounds, therefore it was subjected to column chromatography on silica gel 60, 0.040 – 0.063 (Merck) using a mixture of light petroleum and ethyl acetate 40:60 as the mobile phase. Volume of one fraction was 20 ml. Fractions 25 – 52 were combined and evaporated to dryness and the residue crystallized from anhydrous ethanol. The crystals were filtered off and 0.11 g of colorless needles was obtained. This compound was identified as starting *N′*-hydroxypyrazine-2-carboximidamide using melting point and NMR spectrum.

M.p. 183.5–184.5 °C

^{1}H NMR spectrum (300 MHz, DMSO) δ 10.23 (1H, s, OH), 9.05 (1H, d, *J* = 1.4, H3), 8.64–8.59 (2H, m, H5, H6), 5.95 (2H, bs, NH_2)

^{13}C NMR spectrum (75 MHz, DMSO) δ 148.5, 145.9, 144.5, 143.3, 141.8

4.9 5-methyl-3-(pyrazin-2-yl)-1,2,4-oxadiazole



A stirred mixture of *N′*-hydroxypyrazine-2-carboximidamide (1.38 g; 0.01 mol) and acetic anhydride (1.0 g; 0.01 mol) in 25 ml of xylene was heated under reflux for 3 hours. The xylene was evaporated under reduced pressure and the residue (1.93 g) crystallized from isopropyl alcohol 1.12 g (69%) of off-white small crystals was obtained.

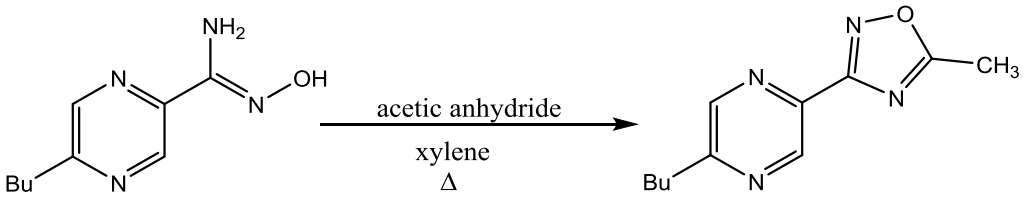
Molecular weight: 162.15 ($C_7H_6N_4O$)
Melting point: 98–99 °C (ref. [91]: 100–104 °C, isopropyl alcohol)

Elemental analysis:

	% C	**% H**	**% N**
Calculated:	51.85	3.73	34.55
Found:	51.95	4.09	34.07

IR spectrum: 3079, 3024 (CH arom.), 2937 (CH aliph), 1589, 1576 (C=N), 1155 (C-O), 918 (N-O)
^{1}H NMR spectrum (500 MHz, DMSO) δ 9.21 (1H, d, *J* = 1.5, H3), 8.86–8.82 (2H, m, H5, H6), 2.71 (3H, s, CH_3)
^{13}C NMR spectrum (500 MHz, DMSO)

4.10 3-(5-butylpyrazin-2-yl)-5-methyl-1,2,4-oxadiazole I



A stirred mixture of 5-butyl-*N*′-hydroxypyrazine-2-carboximidamide (1.94 g; 0.01 mol) and acetic anhydride (1.0 g; 0.01 mol) in 25 ml of xylene was heated under reflux for 3 hours. The xylene was partly evaporated under reduced pressure (approx. 1.33 kPa) and the residue placed to the refrigerator. A small amount of a fine precipitate separated. It was filtered off and dried. A light beige compound (0.20 g) was obtained. Based on NMR results it proved to be a mixture of starting amidoxime with a small amount of the product.

The filtrate contained two distinct compounds, therefore it was subjected to column chromatography.

Chromatographic conditions:

Stationary phase – Silica gel 60 (Merck) 0,040–0,063 mm, 100 g

Mobile phase – light petroleum + ethyl acetate 60:40

Volume of a fraction – 25 ml

Combined fractions 12–19 yielded 1.68 g of the crude product. After crystallization from anhydrous ethanol 0.67 g of an off-white crystals was obtained.

Molecular weight: 218.26 ($C_{11}H_{14}N_4O$)

Melting point: 46.1–46.6 °C

Elemental analysis:

	% C	**% H**	**% N**
Calculated:	60.53	6.47	25.67
Found:	60.42	6.85	25.67

IR spectrum: 2950, 2930, 2869 (CH, aliph.), 1597 (C=N), 1169 (C-O), 912 (N-O)

^{1}H NMR spectrum (300 MHz, DMSO) δ 9.10 (1H, s, *J* = 1.5, H3), 8.73 (1H, s, *J* = 1.5, H6), 2.85 (2H, t, *J* = 7.5 Hz, CH_2), 2.69 (3H, s, CH_3), 1.77–1.61 (2H, m CH_2), 1.41–1.24 (2H, m, CH_2), 0.89 (3H, t, *J* = 7.5, CH_3)

^{13}C NMR spectrum (75 MHz, DMSO) δ 178.2, 166.3, 159.9, 147.7, 142.8, 139.2, 34.4, 30.7, 21.9, 13.8, 12.2

4.11 3-(5-butylpyrazin-2-yl)-5-methyl-1,2,4-oxadiazole II

The reaction was performed as described in the previous case, but directly after the completed heating approx. 15 g of silica gel was added and the mixture evaporated to dryness and subjected to column chromatography.

Chromatographic conditions:
Stationary phase – Silica gel 60 (Merck) 0,040–0,063 mm, 100 g
Mobile phase – light petroleum + ethyl acetate 70:30
Volume of a fraction – 25 ml

Fractions 1–7 contained only the remnants of xylene. Evaporation of fractions 8–12 yielded 2.45 g of the crude product that was dissolved in isopropyl alcohol and left to crystallize in the refrigerator. Only 0.31 g of white crystals was obtained. Their purity was checked by TLC and by determining the melting point. The results were satisfactory and the product was submitted to elemental and spectral analysis.
Molecular weight: 218.26 ($C_{11}H_{14}N_4O$)
Melting point: 47.1–47.6 °C
Elemental analysis:

	% C	**% H**	**% N**
Calculated:	60.53	6.47	25.67
Found:	60.32	6.76	25.44

IR spectrum: 2964, 2950, 2930, 2869 (CH, aliph.), 1597 (C=N), 1168 (C-O), 912 (N-O)
^{1}H NMR spectrum (500 MHz, DMSO) δ 9.10 (1H, d, *J* = 1.5, H3), 8.73 (1H, d, *J* = 1.5, H6) 2.86 (2H, t, *J* = 7.6 Hz, CH_2), 2.70 (3H, s, CH_3), 1.74–1.65 (2H, m CH_2), 1.38–1.28 (2H, m, CH_2), 0.89 (3H, t, *J* = 7.6, CH_3)
^{13}C NMR spectrum (125 MHz, DMSO) δ 178.2, 166.3, 160.0, 147.7, 142.9, 139.2, 34.4, 30.7, 21.9, 13.9, 12.2

The parent liquor after the previous crystallization was subjected to column chromatography once more.

Chromatographic conditions:

Stationary phase – Silica gel 60 (Merck) 0,040–0,063 mm, 100 g
Mobile phase – light petroleum + ethyl acetate 80:20
Volume of a fraction – 25 ml

Evaporation of fractions 7–9 gave 2.07 g of the crude product. After crystallization from isopropyl alcohol 1.28 g of off-white crystals was obtained.
Molecular weight: 218.26 ($C_{11}H_{14}N_4O$)
Melting point: 46.1–46.6 °C
Elemental analysis:

	% C	**% H**	**% N**
Calculated:	60.53	6.47	25.67
Found:	60.04	6.91	25.51

IR spectrum: 2963, 2949, 2929, 2869 (CH-aliph.), 1596 (C=N), 1168 (C-O), 911 (N-O)
^{1}H NMR spectrum (500 MHz, DMSO) δ 9.10 (1H, d, *J* = 1.5, H3), 8.73 (1H, d, *J* = 1.5, H6) 2.86 (2H, t, *J* = 7.6 Hz, CH_2), 2.69 (3H, s, CH_3), 1.73–1.65 (2H, m CH_2), 1.37–1.27 (2H, m, CH_2), 0.89 (3H, t, *J* = 7.6, CH_3)
^{13}C NMR spectrum (125 MHz, DMSO) δ 178.2, 166.3, 160.0, 147.7, 142.9, 139.2, 34.5, 30.8, 21.9, 13.9, 12.2

4.12 Evaluation of in vitro antifungal activity

The antifungal activity of all compounds was evaluated by the modified microdilution broth CSLI standards [95, 96]. The organisms examined included *Candida albicans* ATCC 44859 (American Type Culture Collection, Manassas, VA, USA, **CA**), *Candida tropicalis* 156 (**CT**), *Candida krusei* E 28 (**CK**), *Candida glabrata* 20/I (**CG**), *Trichosporon asahii* 1188 (**TA**), *Aspergillus fumigatus* 231 (**AF**), *Lichtheimia corymbifera* (formerly *Absidia corymbifera*) 272 (**LC**), and *Trichophyton mentagrophytes* 445 (**TM**). All strains tested are clinical isolates obtained from the Department of Clinical Microbiology, University Hospital and Faculty of Medicine, Charles University, Prague, Czech Republic.

Before testing each strain was subcultured on Sabouraud dextrose agar (SDA; Difco/Becton Dickinson, Detroit, MI, USA) and maintained on the same medium at 4 °C. Fungal inocula were prepared by suspending yeasts, conidia, or sporangiospores in sterile 0.85% saline. The cell density was adjusted using a Bürker's chamber to yield a stock suspension of 1.0 ± 0.2 × 105 CFU/mL and 1.0 ± 0.2 × 106 CFU/mL for yeasts and molds, respectively. The final inoculum was made by 1:20 dilution of the stock suspension with the test medium.

The compounds were dissolved in DMSO, and the antifungal activity was determined in RPMI 1640 media (KlinLab, Prague, Czech Republic) buffered to pH 7.0 with 0.165 M 3-morpholinopropane-1-sulfonic acid (Sigma-Aldrich, St. Louis, MO, USA). Controls consisted of medium and DMSO alone. The final concentration of DMSO in the test medium did not exceed 1% (v/v) of the total solution. The concentrations of the studied substances ranged from 500 to 0.488 μmol/L. The minimum inhibitory concentration (MIC), was defined as 80% or greater (for yeasts and yeast-like organisms – IC_{80}), resp. 50% or greater (for molds – IC_{50}) reduction of growth in comparison with the control. The values of MICs were determined after 24 and 48 h of static incubation at 35 °C. In the case of *T. entagrophytes*, the MICs were recorded after 72 and 120 h due to its slow growth rate. Amphotericin B and fluconazole were used as reference antifungal drugs.

Table 1. Minimum inhibitory concentrations of the tested compounds

STRAIN		**COMPOUND – MIC/IC$_{95}$** (μmol.l^{-1})									
		4.2	**4.3**	**4.4**	**4.5**	**4.6**	**4.7**	**4.9**	**4.11**	**AMP B**	**FLU**
CA	*24h*	>500	>500					>500	>500	0.020	0.82
	48h	>500	>500					>500	>500	0.068	1.63
CT	*24h*	>500	>500					>500	>500	0.068	1.63
	48h	>500	>500					>500	>500	0.068	>417.9
CK	*24h*	>500	>500					>500	>500	0.135	52.24
	48h	>500	>500					>500	>500	0.135	104.47
CG	*24h*	>500	>500					>500	>500	0.034	13.06
	48h	>500	>500					>500	>500	0.135	52.24
TA	*24h*	>500	>500					>500	>500	1.082	3.26
	48h	>500	>500					>500	>500	2.164	6.53
AF	*24h*	>500	>500					>500	>500	0.271	>417.9
	48h	>500	>500					>500	>500	0.135	>417.9
LC	*24h*	>500	>500					>500	>500	1.082	>417.9
	48h	>500	>500					>500	>500	2.164	>417.9
TM	*72h*	>500	>500					>500	>500	1.082	26.12
	120h	>500	>500					>500	>500	1.082	52.24

4.2 = *N*′-hydroxypyrazine-2-carboximidamide

4.3 = 5-butyl- *N*′-hydroxypyrazine-2-carboximidamide

4.4 = *N*′-acetoxypyrazine-2-carboximidamide

4.5 = *N*′-acetoxy-5-butylpyrazine-2-carboximidamide

4.6 = *N*′-pivaloyloxypyrazine-2-carboximidamide

4.7 = 5-butyl- *N*′-pivaloyloxypyrazine-2-carboximidamide

4.9 = 5-methyl-3-(pyrazin-2-yl)-1,2,4-oxadiazole

4.11 = 3-(5-butylpyrazin-2-yl)-5-methyl-1,2,4-oxadiazole

4.13 Evaluation of *in vitro* antibacterial activity

The antibacterial activity of all compounds was evaluated by the microdilution broth method [97]. The organisms examined included strains from Czech Collection of Microorganisms (Brno, Czech Republic): *Staphylococcus aureus* CCM 4516/08 (**SA)**, *Escherichia coli* CCM 4517 (**EC)**, *Pseudomonas aeruginosa* CCM 1961 (**PA)**. These strains are recommended as standards for testing of antibacterial activities. Other strains were clinical isolates (Department of Clinical Microbiology, University Hospital and Faculty of Medicine in Hradec Králové, Charles University in Prague, Czech Republic): *Staphylococcus aureus* H 5996/08 (methicilin resistant, **MRSA**), *Staphylococcus epidermidis* H 6966/08 (**SE**), *Enterococcus sp.* J 14365/08 (**EF**), *Klebsiella pneumoniae* D11750/08 (**KP**), *Klebsiella pneumoniae* J 14368/08 (ESBL positive, **KP-E**). All strains were subcultured on Mueller-Hinton agar (MHA) (Difco/Becton Dickinson, Detroit, MI) at 35 °C and maintained on the same medium at 4 °C. Prior to testing, each strain was passaged onto MHA. Bacterial innocula were prepared by suspending in sterile 0.85% saline. The cell density of the inoculum was adjusted using densitometer to yield suspension of density equivalent 0,5 McFarland scale which is equal to a number of 1. 5×10^8 viable colony forming units (CFU)/mL.

The compounds were dissolved in DMSO, and the antibacterial activity was determined in Mueller-Hinton liquid broth (Difco/Becton Dickinson, Detroit, MI), buffered to pH 7.0. Controls consisted of medium and DMSO alone. The final concentration of DMSO in the test medium did not exceed 1% (v/v) of the total solution composition. The minimum inhibitory concentration (MIC), defined as 95% inhibition of bacterial growth as compared to control, was determined after 24 and 48 h of static incubation at 35 °C. Penicillin and ciprofloxacin have been used as reference antibacterial drugs.

Table 1. Minimum inhibitory concentrations of the tested compounds

STRAIN		**COMPOUND – MIC/IC$_{95}$** (μmol.l^{-1})									
		4.2	**4.3**	**4.4**	**4.5**	**4.6**	**4.7**	**4.9**	**4.11**	**PEN**	**CIPR**
SA	*24h*	>500	>500					>500	>500	0.24	0.98
	48h	>500	>500					>500	>500	0.24	0.98
MR SA	*24h*	>500	>500					>500	>500	125	500
	48h	>500	>500					>500	>500	125	500
SE	*24h*	>500	>500					>500	>500	31.25	250
	48h	>500	>500					>500	>500	125	250
EF	*24h*	>500	>500					>500	>500	7.81	0.98
	48h	>500	>500					>500	>500	15.62	0.98
EC	*24h*	>500	>500					>500	>500	125	0.06
	48h	>500	>500					>500	>500	125	0.06
KP	*24h*	>500	>500					>500	>500	250	0.12
	48h	>500	>500					>500	>500	500	0.12
KP-E	*24h*	>500	>500					>500	>500	>500	>500
	48h	>500	>500					>500	>500	>500	>500
PA	*72h*	>500	>500					>500	>500	>500	3.9
	120h	>500	>500					>500	>500	>500	7.81

4.2 = *N′*-hydroxypyrazine-2-carboximidamide

4.3 = 5-butyl- *N′*-hydroxypyrazine-2-carboximidamide

4.4 = *N′*-acetoxypyrazine-2-carboximidamide

4.5 = *N′*-acetoxy-5-butylpyrazine-2-carboximidamide

4.6 = *N′*-pivaloyloxypyrazine-2-carboximidamide

4.7 = 5-butyl- *N′*-pivaloyloxypyrazine-2-carboximidamide

4.9 = 5-methyl-3-(pyrazin-2-yl)-1,2,4-oxadiazole

4.11 = 3-(5-butylpyrazin-2-yl)-5-methyl-1,2,4-oxadiazole

5. Discussion

My work was concetrated on amidoximes and their *O*-acylated and cyclic derivatives. The investigation of the amidoxime derivatives is of great importance in developing a range of new drugs. The theoretical part deals with methods of the acylation of amidoximes in general, acylation using anhydrides and acyl chlorides, and finally various methods of cyclization to 3,5-disubstituted-1,2,4-oxadiazoles were described.

As far as the experimental part is concerned, preparation of following pyrazine amidoximes and derivatives was attemped:

- 5-butylpyrazine-2-carbonitrile (precursor of pyrazine amidoxime, 4.1)
- *N*′-hydroxypyrazine-2-carboximidamide (4.2)
- 5-butyl-*N*′-hydroxypyrazine-2-carboximidamide (4.3)
- *N*′-acetoxypyrazine-2-carboximidamide (4.4)
- *N*′-acetoxy-5-butylpyrazine-2-carboximidamide (4.5)
- *N*′-pivaloyloxypyrazine-2-carboximidamide (4.6)
- 5-butyl-*N*′-pivaloyloxypyrazine-2-carboximidamide (4.7)
- 3-[({[amino(pyrazin-2-yl)methylidene]amino}oxy)carbonyl]pyrazine-2-carboxylic acid (4.8)
- 5-methyl-3-(pyrazin-2-yl)-1,2,4-oxadiazole (4.9)
- 3-(5-butylpyrazin-2-yl)-5-methyl-1,2,4-oxadiazole (4.10 and 4.11)

The first synthetised compound was 5-butylpyrazine-2-carbonitrile from commercially available pyrazinecarbonitrile. It was done in accordance with Opletalová *et al.* [93].

N′-hydroxypyrazine-2-carboximidamide and 5-butyl-*N*′-hydroxypyrazine-2-carboximidamide were synthesized according to Gezginci *et al*. [98]. 5-butyl-*N*′-hydroxypyrazine-2-carboximidamide is a novel compound.

The procedure reported by Pancechowska-Ksepko *et al.* [71] was used for the preparation of the esters – *N*′-acetoxypyrazine-2-carboximidamide and *N*′-acetoxy-5-butylpyrazine-2-carboximidamide. The melting point of the former ester was notably decreased in comparison with the data found in literature, but its purity was confirmed by the CHNS analysis. The difference in melting points could be due to different solvents used for the crystallization. Whilst the Polish authors used benzene, our product was crystallized from anhydrous ethanol. The latter ester is a novel compound that has not been reported in literature so far.

I have also tried to use the same procedure for the acylation of *N*′-hydroxypyrazine-2-carboximidamide with the anhydride of 2,3-pyrazinedicarboxylic anhydride. Unfortunately, this attempt was unsuccessful, and only starting amidoxime was isolated from the reaction mixture. The failure can be a result of poor solubility of 2,3-pyrazinedicarboxylic anhydride in

toluene since precipitation of a solid was observed immediately after the addition of the anhydride to the solution of amidoxime. Therefore, it will be necessary to find a suitable solvent and perform the reaction once more. As 2,3-pyrazinecarboxylic acid is a dicarboxylic acid, using 2 eq. of amidoxime for 1 eq. of the anhydride could result in the desired product.

The final experiments were focused on the preparation of 3,5-disubstituted-1,2,4-oxadiazoles by a process patented by Fanshawe and Safir [91]. Firstly, 5-methyl-3-(pyrazin-2-yl)-1,2,4-oxadiazole was obtained successfully without the necessity to use column chromatography (part 4.9). Afterwards, during the attempt to produce 3-(5-butylpyrazin-2-yl)-5-methyl-1,2,4-oxadiazole (part 4.10), the product contained both starting amidoxime and the desired product. Therefore, column chromatography was performed to separate the oxadiazole. Separation was succesfull, but the crystallization from ethanol yielded only 0.67 g (31%) of the product.

To optimize conditions for the separation and purification of the product, the experiment was performed once more using a slightly modified procedure. However, the amount of the demanded product after crystallization from isopropyl alcohol was found low as well – only 0.31 g (14%). That is why mother liquid was submitted also to column chromatography. After the column chromatography and crystallization from isopropyl alcohol 1.28 g (59%) of 3-(5-butylpyrazin-2-yl)-5-methyl-1,2,4-oxadiazole was obtained. Total yield of the modified procedure 4.11 was 1.59 g (73%).

Due to the presence of the double bond, amidoximes can have *E*- or *Z*-configuration. In the NMR spectra of amidoximes prepared within this thesis, only one set of signals was found. Hence, only one of the possible isomers was present in the DMSO solution. It is most probably *Z*-isomer since according to the data reported in literature, unsubstituted and monosubstituted amidoximes exist as *Z*-isomers in both crystalline state and solutions [99–101]. Also, in the spectra of acylated amidoximes only one set of signals was observed.

Furthermore, amidoximes and their derivatives were subjected to biological assay in order to evaluate their *in vitro* antifungal and antibacterial properties. Unfortunatelly, the results of the assays for amidoximes and 1,2,4-oxadiazoles were unsatisfactory. For the esters, biological results will be available later.

6. Conclusions

During my diploma thesis, reaction conditions for *O*-acylation of amidoximes and formation of 1,2,4-oxadiazoles were examined.

Acylation of amidoximes with acetic anhydride and pivalic anhydride led to corresponding acylated product.

Acylation of N′-hydroxypyrazine-2-carboximidamide which theoretically could yield 3-[({[amino(pyrazin-2-yl) methylidene]amino}oxy)carbonyl] pyrazine-2-carboxylic acid, was not successful.

In the end, oxadiazole preparation by heating the amidoximes in xylene gave 5-methyl-3-(pyrazin-2-yl)-1,2,4-oxadiazole without further purification by column chromatography. On the other hand, in case of 3-(5-butylpyrazin-2-yl)-5-methyl-1,2,4-oxadiazole column chromatography was needed to separate the product from the starting 5-butylpyrazine amidoxime and remnants of xylene.

Although amidoximes and oxadiazoles were not active against fungi and bacteria, further investigation is required for esters and other derivatives of amidoximes.

7. References

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